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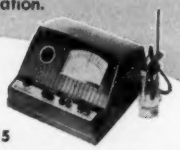

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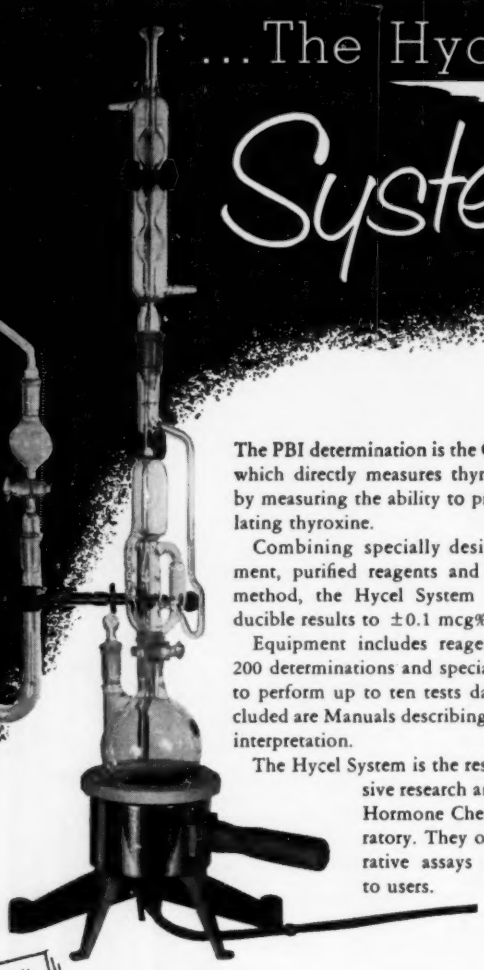

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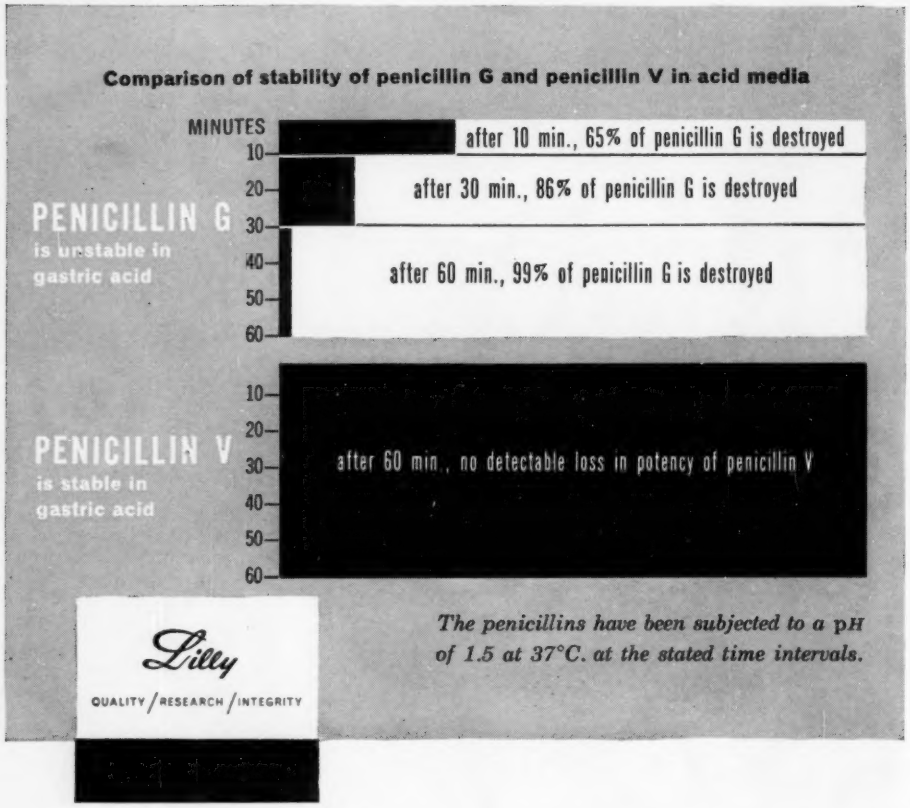
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THE METABOLISM OF AMMONIA AND α-KETO-ACIDS IN LIVER DISEASE AND HEPATIC COMA [1]

By W. H. J. SUMMERSKILL,[2] STANLEY J. WOLFE,[3] AND CHARLES S. DAVIDSON

(From the Thorndike Memorial Laboratory and Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.)

(Submitted for publication August 20, 1956; accepted November 13, 1956)

A causal relationship between ammonia intoxication and hepatic coma is suggested by the reproduction of the syndrome of impending hepatic coma in sensitive patients with liver disease by substances from which ammonia can be derived (1) and is supported by demonstration of deranged ammonia metabolism in liver disease (2). The frequent finding of elevated peripheral vein ammonia concentrations in hepatic coma (3) has encouraged incrimination of ammonia in the genesis of the syndrome (4, 5). Conversely, reservations have been expressed that such results may signify no more than impaired nitrogen metabolism secondary to liver disease (6, 7). More recently Bessman and Bessman (8) have questioned the validity of observations based on peripheral vein blood alone by demonstrating significant arterio-venous ammonia difference in hepatic coma. Moreover, a close relationship has been reported between arterial concentrations and neurological status in a patient with this complication (9).

The purposes of this paper are: 1) Further assessment of the significance of arterial concentrations and A-V ammonia differences in hepatic coma, directing particular attention to the effect of eliminating from the diet nitrogenous substances from which ammonia may be derived; and 2) Investigation of a possible relationship between disordered ammonia metabolism and the elevated blood pyruvate and α-ketoglutarate concentrations reported in liver disease (10, 11). Arterial concentration and tissue metabolism of ammonia have been compared with the neuropsychiatric state and with the protein intake in uncomplicated liver disease and hepatic coma. Simultaneous estimations of blood pyruvate and α-ketoglutarate were performed. The relationship of the keto-acids to ammonia metabolism was further studied in a smaller group of patients with liver disease by measuring blood concentrations of these substances in response to the administration of ammonium chloride.

MATERIAL AND METHODS

Patients. Twenty-seven patients (16 male and 11 female) in impending hepatic coma or coma were studied. Ages were distributed between 29 and 70 years. Twenty patients had cirrhosis associated with chronic alcoholism. The etiology of cirrhosis was uncertain in three patients. One patient had hemochromatosis and another Wilson's disease. One patient had an hepatic lymphoma and the remaining patient had carbon tetrachloride poisoning. Liver failure, evident from progressive jaundice and severe ascites, was judged the basis of hepatic coma in 16 patients. Other factors precipitating coma included major gastrointestinal hemorrhage (5 patients), acute pyogenic infections (3 patients), intolerance of nitrogenous substances with deterioration of liver function (2 patients with portacaval anastomoses), paracentesis abdominis (1 patient) and a major surgical operation (1 patient).

Eleven patients with cirrhosis in the absence of hepatic coma were also studied (10 chronic alcoholics and 1 in whom the etiology of liver disease was uncertain). A group of 18 control subjects without evidence of hepatic, renal or metabolic disorder was recruited from hospital patients and staff.

The diagnosis of liver disease was made on clinical and biochemical grounds; histological confirmation from biopsy or autopsy specimens was available in 27 patients, including 18 of the 20 patients who died in hepatic coma.

Neuropsychiatric assessment. Patients in coma (including impending hepatic coma) were examined daily or more often, assessment being based on the clinical syndrome reported by Adams and Foley (12) and assisted by EEG records when necessary. The charac-

[1] This work was supported in part by a contract between Harvard University and the Office of The Surgeon General, Department of the Army, and in part by grants to Harvard University from Merck & Co., Inc., Rahway, New Jersey, The Nutrition Foundation, Inc., New York, New York, and Lederle Laboratories Division of the American Cyanamid Company, Pearl River, New York.

[2] Rockefeller Travelling Fellow of the Medical Research Council of Great Britain.

[3] Public Health Service Research Fellow of the National Heart Institute.

TABLE I

Cerebral (arterial-jugular bulb difference) and peripheral (arterial-peripheral vein difference) uptake and release of ammonia during terminal two days of hepatic coma *

	Patient and diagnosis	Serum bilirubin (*mg./100 ml.*)	Ascites (0–+++)	Arterial NH_3N (*μg./100 ml. blood*)	A-V difference Ammonia "uptake" (+) or "release" (−) Cerebral (*μg./100 ml.*)	Peripheral (*μg./100 ml.*)
GA	Cirrhosis of the alcoholic	26.0	+++	275	+15	+61
SU	Cirrhosis of the alcoholic, massive gastrointestinal hemorrhage	18.4	+++	60	+12	+16
ST	Cirrhosis of the alcoholic, massive gastrointestinal hemorrhage	2.9	+++	250	+66	+83
BC	Cirrhosis of the alcoholic, lobar pneumonia	6.4	0	271	+4	+1
MC	Cirrhosis of the alcoholic	11.0	+++	171	−75	−97
GE	Wilson's Disease	1.9	+++	229	−90	+42
BE	Cirrhosis of the alcoholic, massive gastrointestinal hemorrhage	23.6	++	354		+4
EA	Cirrhosis of the alcoholic	22.0	+++	262		−1
MG	Cirrhosis of the alcoholic	19.0	++	200		−11
MS	Hepatic lymphoma, gastrointestinal hemorrhage	0.5	0	114		−4
SP	Carbon tetrachloride poisoning	38.0	+	52		−11
MK	Cirrhosis of the alcoholic	33.1	+++	160		+63

* Although in coma, the patients were not moribund.

teristic fluctuation and frequent disparity between psychiatric and objective neurological findings permitted an accurate distinction only between impending coma and coma for the purpose of this report, stuporous patients responding only to strong stimuli being placed in the latter category.

Protein intake. Protein intake, during or immediately prior to hepatic coma arising in hospitalized patients, was assessed from the ward diet or calculated from the restricted protein diet, usually a low-sodium milk product,[4] on which patients were maintained. Progressive or advanced hepatic coma was treated with exclusion of all nitrogenous substances (protein, drugs, etc.) from the diet and with broad spectrum antibiotics by mouth (7). Chlortetracyclene in doses of 2 to 4 gm. daily was administered for the duration of the neurological syndrome. The possibility of gastrointestinal bleeding was checked by inspection of feces, examination of stools for occult blood and, in fatal cases, at autopsy.

Biochemical methods. Blood ammonia was determined by a modification (13) of Conway's method (14); blood pyruvate and α-ketoglutarate were estimated using the method of Seligson and Shapiro (15). Blood was drawn without stasis into specially cleaned syringes and was introduced into the Conway units or flasks at the bedside. The accuracy of these methods in our hands, calculated from the standard deviation from mean recoveries, was ± 3 per cent for ammonia, using standards and blanks, ± 6 per cent for α-ketoglutarate and ± 9 per cent for pyruvate. The recoveries for keto-acids were carried out using human blood. "Uptake" of ammonia or keto-acids by brain or peripheral tissues was assumed when the differences between concentrations in arterial and appropriate venous blood (A-V difference) were positive and, conversely, release is represented by negative differences.

Administration of ammonium chloride. Alterations in blood ammonia, pyruvate and α-ketoglutarate concentrations following the administration of ammonium chloride were measured in 5 patients with liver disease, whose clinical and biochemical findings are given in Table III, and in 4 control subjects. Three other control subjects received ammonium chloride prior to determination of arterial and peripheral vein ammonia concentrations. Ammonium chloride was given by mouth (as non-enteric coated capsules) or intravenously (2 per cent solution in water or sodium chloride infused over 45 minutes) in 3.0 or 4.0-gm. doses. Individuals with severely impaired liver function, extensive portal-systemic collateral venous systems or previous evidence of neuropsychiatric sensitivity to nitrogenous substances received the smaller amount by mouth in view of their susceptibility to hepatic coma induced by ammonium salts (1). Control subjects were given ammonium chloride intravenously as use of the oral route would have prevented most of the ammonia from reaching the peripheral tissues owing to its removal from portal blood by a healthy liver (2). Blood specimens (arterial, peripheral venous, or both) were taken immediately prior to administration of the salt and at varied 30-minute intervals up to four hours for determination of blood ammonia, pyruvate and α-ketoglutarate concentrations. Values are reported at 0, 30 to 60, 90 to 120 and 180 to 240 minutes, representing actual readings or mean levels where more than one estimation was made in the relevant period.

[4] Lonalac®, Mead Johnson and Company, Evansville, Indiana.

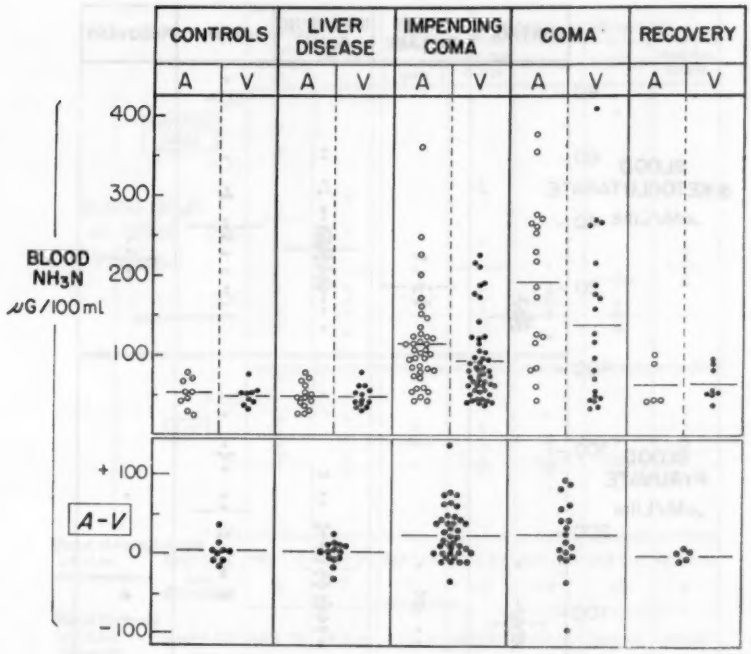

FIG. 1. ARTERIAL (A) AND PERIPHERAL VEIN (V) AMMONIA CONCENTRATIONS WITH A–V DIFFERENCES IN CONTROL SUBJECTS AND PATIENTS WITH LIVER DISEASE WITH AND WITHOUT HEPATIC COMA

RESULTS

Blood ammonia and keto-acid concentrations in hepatic coma

Comparisons were made between the arterial and peripheral vein ammonia concentrations and A-V difference of ammonia in the control group and in patients with liver disease and hepatic coma (Figure 1). Control subjects and patients with liver disease without coma had similar fasting values of ammonia in arterial and venous blood (upper limit of normal 75 μg. per 100 ml.) and A-V differences in both groups indicated that a small and variable uptake or release of ammonia by peripheral tissues occurred in the fasting state. During impending hepatic coma the mean arterial concentration (113 μg. per 100 ml.) was elevated, but a quarter of the readings remained within the normal range. The mean concentration was lower in the peripheral vein (92 μg. per 100 ml.), a half of the values being within normal limits. These findings were associated with a greater positive A-V difference of ammonia in peripheral tissues in the majority of cases. In patients who had progressed to coma, higher mean values of arterial and peripheral vein ammonia were found (193 and 139 μg. per 100 ml., respectively) with a smaller proportion of readings (about 10 per cent arterial and 25 per cent venous) still remaining in the normal range. The A-V difference was still predominantly positive but relative equilibrium was not uncommon and tissue release of ammonia, occasionally of a high degree, was sometimes observed. Values obtained from patients in the phase of recovery, but still exhibiting residual neuropsychiatric disorder, showed a return to normal blood ammonia concentrations and tissue equilibrium of ammonia.

Blood α-ketoglutarate and pyruvate values were determined simultaneously with ammonia (Figure 2). Mean concentrations of both substances in fasting patients with liver disease (α-ketoglutarate 20.7, pyruvate 112 μM per liter) were above mean control values (α-ketoglutarate 11.5, pyru-

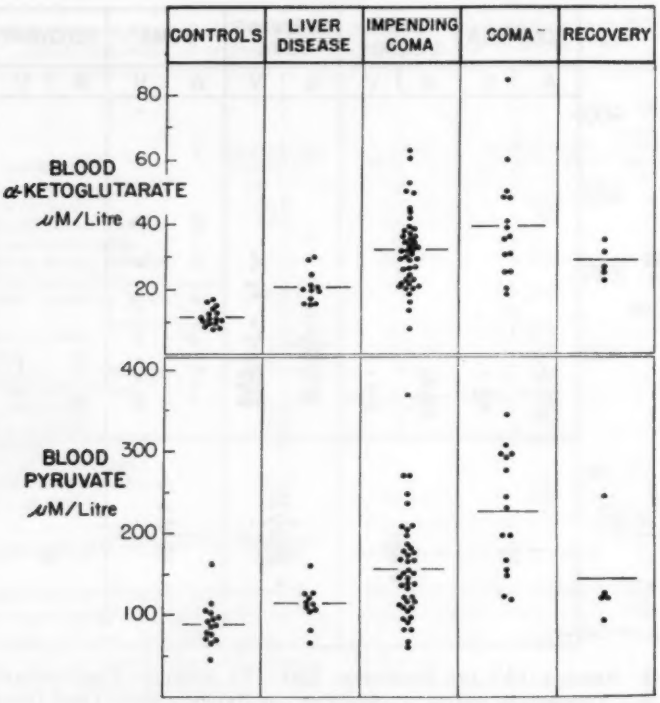

FIG. 2. BLOOD α-KETOGLUTARATE AND PYRUVATE CONCENTRATIONS IN CONTROL SUBJECTS AND PATIENTS WITH LIVER DISEASE WITH AND WITHOUT HEPATIC COMA

vate 89 μM per liter), but there was a considerable overlap in pyruvate values. Progressive increase in mean values in impending coma (α-ketoglutarate 32.3, pyruvate 154 μM per liter) and coma (α-ketoglutarate 39.7, pyruvate 225 μM per liter) was observed, but the scatter was wide. A third of the α-ketoglutarate and half of the pyruvate values during impending coma and coma were below the upper limits found in patients with uncomplicated liver disease.

The influence of nitrogenous material in the intestines on blood ammonia concentration in hepatic coma

Blood ammonia concentrations during hepatic coma were studied in relation to nitrogenous material in the intestine (dietary protein, gastrointestinal bleeding, drugs such as ammonium chloride, etc.) (Figure 3). The highest arterial ammonia concentrations occurred when coma was precipitated by gastrointestinal hemorrhage or by intolerance to nitrogenous substances and the mean levels in patients on conventional home or ward diets exceeded that found when coma was associated with low protein feeding. Only one value in each group was within the normal range. During treatment by total protein withdrawal and antibiotics, two-thirds of arterial ammonia readings remained high during the first 48 hours, but there was a striking decline towards normal in all patients during the second to fifth-day period, although only three values fell within the normal range. In the sixth to tenth-day period, the majority of arterial ammonia concentrations remained at or near normal, but a rise occurred in some patients and a further elevation in the mean value was observed in patients who survived on this regimen for more than 10 days, in the absence of protein feeding, gastrointestinal hemorrhage or uremia. Those who recovered and the fatal cases

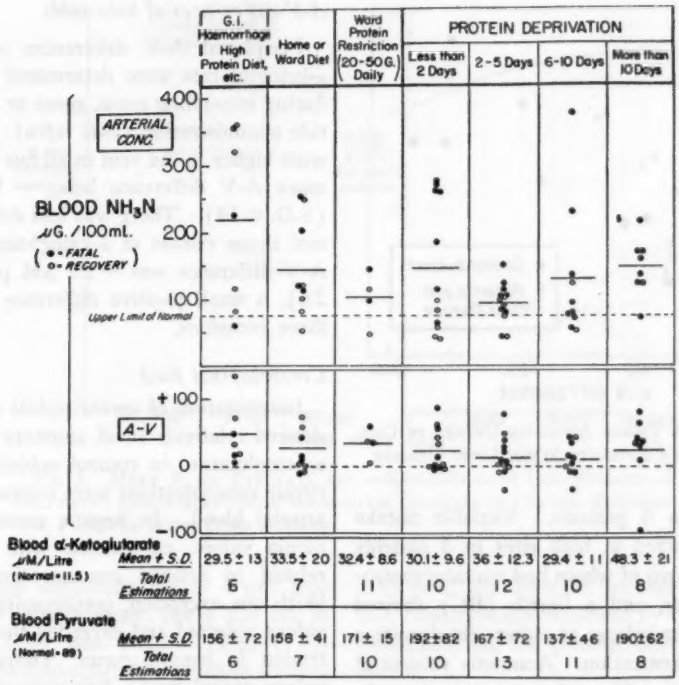

FIG. 3. ARTERIAL AMMONIA CONCENTRATIONS, A-V AMMONIA DIFFERENCES AND BLOOD α-KETOGLUTARATE AND PYRUVATE CONCENTRATIONS DURING HEPATIC COMA IN RELATION TO NITROGENOUS MATERIAL IN THE GASTROINTESTINAL TRACT

had arterial ammonia concentrations which were comparable in the early stages, but in patients who survived they returned towards normal more rapidly after protein withdrawal.

It was not possible to demonstrate a similar relationship between blood pyruvate or α-ketoglutarate concentrations and intake of nitrogenous material (Figure 3). Mean values for both substances were high at all stages, particularly in patients who survived more than 10 days, but the scatter was wide.

Uptake of ammonia by peripheral tissue and brain

Peripheral A-V ammonia difference in relation to arterial concentrations greater than 100 μg. per 100 ml. was compared in control subjects who had received ammonium chloride to patients with liver disease who also had received ammonium chloride or were in the early phase of impending hepatic coma (Figure 4). Although uptake of ammonia occurred in both groups, it was greater in control subjects in relation to arterial concentration and the impaired uptake in patients with liver disease was particularly striking at ammonia concentrations greater than 200 μg. per 100 ml. No difference was observed between patients with uncomplicated liver disease and those in impending hepatic coma.

Uptake of ammonia was the usual finding during the course of hepatic coma (Figures 1 and 3) and was related to arterial concentration. Tissue equilibrium or release of ammonia despite high arterial concentrations was not infrequent, however, and these findings were mainly limited to the terminal phase of coma. The arterial concentrations and A-V ammonia differences in 12 unconscious, but not moribund, patients who were studied during the ultimate 2 days of coma are reported in Table I. Cerebral and peripheral A-V differences of ammonia were determined

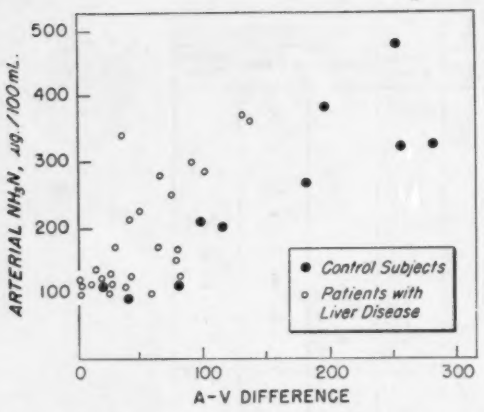

FIG. 4. PERIPHERAL TISSUE AMMONIA UPTAKE IN CONTROL SUBJECTS AND PATIENTS WITH LIVER DISEASE

simultaneously in 6 patients. Variable uptake of ammonia occurred at both sites in 3 patients (GA, SU, ST), two of whom had massive gastrointestinal bleeding, and a fourth (BC) showed neither uptake nor release at either site despite a high arterial concentration. Ammonia release of a high degree was taking place from both brain and peripheral tissues in the fifth patient (MC) and the remaining subject (GE) exhibited release of cerebral ammonia associated with peripheral uptake. Of the other 6 patients, in whom peripheral studies alone were performed, only one (MK) had a positive A-V difference, the remainder showing ammonia equilibrium, although arterial concentrations were high in all but one instance.

A-V differences of keto-acids

Peripheral A-V differences of pyruvate and α-ketoglutarate were determined on 14 occasions during impending coma, coma or ammonium chloride administration (*vide infra*). Pyruvate levels were higher in the vein in all but one instance, the mean A-V difference being − 15 μM per liter (S.D. ± 14). There was less evidence of peripheral tissue release of α-ketoglutarate. The mean A-V difference was − 2.1 μM per liter (S.D. ± 2.6), a small positive difference being found on three occasions.

Cerebrospinal fluid

Investigation of cerebrospinal fluid (Table II) showed relatively small amounts of ammonia and α-ketoglutarate in control subjects, although pyruvate concentrations were comparable to those in arterial blood. In hepatic coma very high ammonia values, comparable with but not clearly related to arterial concentrations, were found. With one exception, cerebrospinal fluid pyruvate values reflected and exceeded the arterial concentration in hepatic coma. Despite high arterial values, spinal fluid α-ketoglutarate concentrations remained relatively low, but a linear increase with arterial values occurred. High pyruvate and α-ketoglutarate values coincided in blood and cerebrospinal fluid but were unrelated to ammonia concentrations at either site.

Blood keto-acid concentrations in response to ammonium chloride administration

Control subjects demonstrated no constant alteration in mean blood α-ketoglutarate concentra-

TABLE II

Arterial and cerebrospinal fluid concentrations of ammonia, pyruvate and α-ketoglutarate in control subjects and in terminal hepatic coma

	Ammonia (NH_3N μg./100 ml.)		Pyruvate (μM/Liter)		α-Ketoglutarate (μM/Liter)	
	Arterial	C.S.F.	Arterial	C.S.F.	Arterial	C.S.F.
Controls (7 subjects)						
Mean ± S.D.	52	20 ± 6*	112	107 ± 25	11.5	1.1 ± 0.8
Hepatic coma						
Patient M. C.	171	408	295	275	85.5	6.5
R. Y.	122	161	190	218	36.5	3.9
B. E.	71	76	111	206	34.5	2.6
G. E.	229	92	92	123	27.7	2.0

* From Clarke, Parsons Smith, Sherlock, and Summerskill (29).

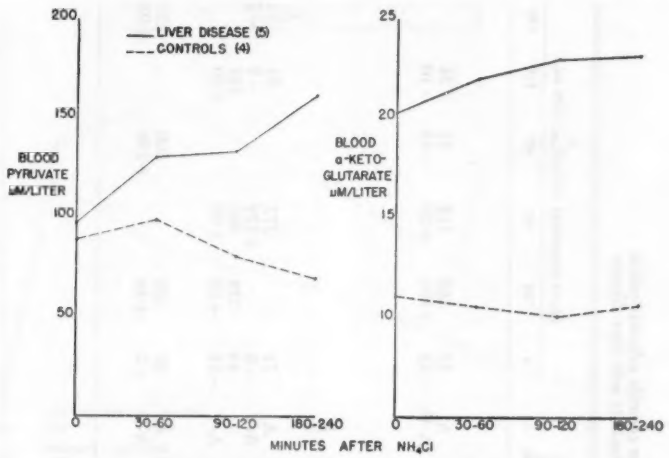

FIG. 5. MEAN BLOOD PYRUVATE AND α-KETOGLUTARATE CONCENTRATIONS FOLLOWING ADMINISTRATION OF AMMONIUM CHLORIDE TO CONTROL SUBJECTS AND PATIENTS WITH LIVER DISEASE

tions following the administration of 4 gm. ammonium chloride intravenously, but a late fall in mean pyruvate level was evident (Figure 5). Similarly, individual α-ketoglutarate values showed little variation from the fasting reading in this group, but pyruvate concentrations, after an early increase in 2 patients, declined as the test continued (Table III). All patients with liver disease exhibited a rise of pyruvate and α-ketoglutarate concentration both in mean values (Figure 5) and individual readings (Table III). The more striking elevations occurred in pyruvate levels which progressed steadily to a maximum at the 3 to 4-hour determination. One patient (B. A.), in whom sensitivity was anticipated owing to chronic nitrogen intolerance following portacaval anastomosis, showed a 95 per cent increase in pyruvate concentration after only 3.0 gm. of ammonium chloride by mouth, but other patients receiving the drug by the oral route (S.E., T.A.) exhibited smaller augmentations of blood pyruvate values than those receiving intravenous ammonium chloride (B.U., C.O.), despite comparable or worse liver function.

The mean elevation of blood α-ketoglutarate concentrations in patients with cirrhosis in response to ammonium chloride was less, but of a magnitude beyond the error of the method. It was a constant finding in every patient (Table III), and the greatest increase of α-ketoglutarate concentration occurred by 30 to 60 minutes, thus preceding the greatest alteration in pyruvate values.

Ammonia uptake by peripheral tissues following the administration of ammonium chloride was demonstrated by elevation of blood ammonia levels and positive A-V ammonia differences in both groups (Table III), coinciding in patients with liver disease with elevation of α-ketoglutarate and preceding the major rise in pyruvate levels. The return to fasting values occurred earlier in patients receiving the drug intravenously, but further assessment of individual tolerance was not considered relevant to the study.

The sequence of events is demonstrated in Figure 6. Following oral ammonium chloride, rapid elevation of blood ammonia levels was accompanied by a rise in peripheral tissue uptake of ammonia and α-ketoglutarate concentration. The steady rise of blood pyruvate values continued after blood levels of ammonia and α-ketoglutarate had declined and after the patient returned to his initial neuropsychiatric state.

DISCUSSION

Increased peripheral vein ammonia concentrations reported in patients with liver disease uncomplicated by hepatic coma (2, 3, 5, 7, 16) are

TABLE III

*Blood pyruvate, α-ketoglutarate and ammonia concentrations following * ammonium chloride administration (4.0 gm. intravenous infusion unless otherwise indicated) in control subjects and patients with liver disease*

Patient, Age, Sex	Serum bilirubin (*mg.%*)	Ascites	Impending coma	Blood pyruvate (*μM/Liter*) 0	30–60	90–120	180–240	Blood α-ketoglutarate (*μM/Liter*) 0	30–60	90–130	180–240		Blood ammonia† (NH_3N *μg./100 ml.*) 0	30	60	90	120	180
Control subjects																		
A. N., 29, F				86	62	55	53	10.5	8.2	8.8	9.9							
S. I., 46, M				66	134	80	81	8.1	10.7	8.6	9.7	A	27	388	118	44	34	
												A-V	−15	+196	+20	−18	−10	
F. L., 52, M				114	97	103	66	16.0	13.7	13.7	13.5							
C. R., 66, M				92	105	85	79	10.0	9.4	9.4	9.4							
			Mean	89	99	81	72	11.1	10.5	10.1	10.6							
Liver disease																		
B. U., 44, M	9.0	Transient	0	126	195	198	241	29.8	32.2	32.2	33.3	A	71		373		71	71
												A-V	−6		+129		−9	−5
T. A.,‡ 46, F	1.0	+++	0	81	95	113	135	17.9	19.0	21.4	23.2	A	43	279	302		167	
												A-V	−14	+66	+90		+80	
C. O., 61, M	1.0	0	0	67	78	71	112	14.4	15.6	17.9	18.1							
B. A.,§ 65, M	6.0	0	Recurrent	118	160	180	206	22.8	24.9	26.9	23.3							
S. E.,§ 70, M	3.0	0	Transient	92	123	110	110	16.9	19.8	17.5	18.4	A	89	320		360		284
												A-V	+2	+34		+136		+98
			Mean	97	130	134	161	20.3	22.2	23.2	23.3							

* Time in minutes.
† A—Arterial, A-V—Arterial-Peripheral Vein Difference.
‡ Ammonium chloride, 4.0 gm. by mouth.
§ Ammonium chloride, 3.0 gm. by mouth.

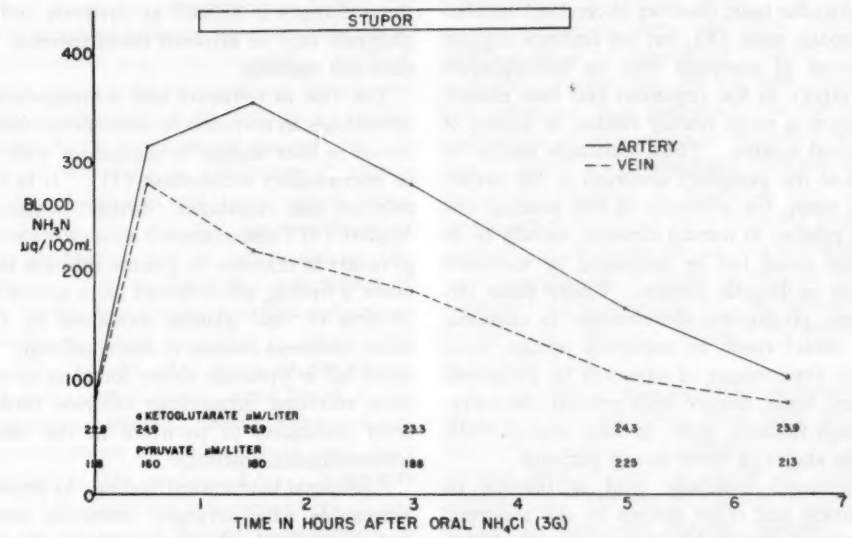

FIG. 6. PERIPHERAL TISSUE UPTAKE OF AMMONIA (A–V DIFFERENCE) AND BLOOD KETO-ACID CONCENTRATIONS DURING EPISODE OF IMPENDING HEPATIC COMA PRECIPITATED BY AMMONIUM CHLORIDE

variably affected by fasting (17, 18), but in our patients both arterial and venous values were normal under such circumstances. Impaired ammonia tolerance in liver disease (2), however, permits abnormal augmentations of blood ammonia following the ingestion of protein (19), blood (20), and other nitrogenous material (1). The highest ammonia concentrations in coma itself were also found by us to be related to these factors. During treatment with protein withdrawal and oral broad spectrum antibiotics, which presumably reduces the formation of ammonia and other toxic substances by suppression or change of intestinal flora (7, 21, 22), arterial ammonia concentrations fell towards, but seldom to, normal. Simultaneously, clinical improvement often occurred and the decline in arterial ammonia values was earlier and greater in patients who subsequently recovered.

The finding of rising arterial ammonia concentrations later in the course of fatal cases, despite continued treatment and the absence of gastrointestinal bleeding or uremia, could only sometimes be related to release of ammonia from peripheral tissues or brain. Possible additional sources of ammonia include the kidneys, which function abnormally in hepatic coma (23), and the failing liver, as high ammonia levels have been reported in renal and hepatic vein blood in hepatic coma (7, 16). The parent compound is unknown and extensive investigation of the phenomenon of ammonia release from mouse brain failed to identify its origin (24, 25). It is relevant, however, that Diamox® may liberate ammonia from the brain by direct inhibition of enzyme function, with elevation of arterial ammonia levels and the production of impending hepatic coma (18), and it also releases ammonia from the kidney into the renal vein (26).

The high incidence of elevated arterial ammonia concentrations in hepatic coma, with a terminal rise in fatal cases or a prompt fall on recovery, supports incrimination of ammonia intoxication in the genesis of the condition. However, the relationship between clinical status, arterial ammonia concentrations and ammonia uptake by brain and muscle reported by others (8, 9) is at variance with the frequently poor correlation in our patients. The occasional finding of normal values in coma also demands consideration.

Ammonia uptake by the brain, resulting in depletion of available α-ketoglutarate (27), has been

suggested as the basic disorder of cerebral metabolism in hepatic coma (8), but our findings suggest that removal of ammonia may be accomplished without injury to the organism and that clinical deterioration is more readily related to failure of this removal system. Thus, although uptake of ammonia at the periphery occurred in the earlier stages of coma, the efficiency of this process was impaired relative to normal capacity, usually to an extent that could not be explained by increased blood flow in hepatic disease. Under these circumstances, progressive deterioration in ammonia removal would result in negligible uptake, equilibrium or even release of ammonia by peripheral tissues and brain, despite high arterial concentration. Such findings were, in fact, characteristic of the late stages of coma in our patients.

An increased metabolic load is thrown on brain, muscle and other tissues by the abnormal and prolonged rises in blood ammonia which follow absorption of ammonia from the portal vein in some patients with liver disease (2). This may explain the impairment of peripheral ammonia uptake which was found even in the absence of hepatic coma, and stresses the importance of these adjuvant sites of ammonia removal. The pre-existing efficiency of these pathways may therefore determine the onset of coma in some instances, and minor overloading could account for the personality changes (28) and EEG abnormalities (29) sometimes observed in liver disease in the absence of objective neurological changes. The threshold of coma, depending on the magnitude of the insult and previous efficiency of cerebral and peripheral tissue ammonia removal, is therefore unlikely to be closely related to blood ammonia concentrations, which reflect ammonia derived from the gastrointestinal tract modified by endogenous uptake and release at various sites.

Evidence of derangement of intermediary metabolism by ammonia was obtained by the administration of ammonium chloride to patients with liver disease. The elevation of blood pyruvate and α-ketoglutarate in response to this procedure suggests that the high keto-acid values found in hepatic coma were also related to impaired ammonia metabolism, although imperfect metabolism by a failing liver (30) may be an additional factor. Biochemical interpretation of these changes is difficult as pyruvate and α-ketoglutarate may be involved simultaneously in more than one reaction.

The rise in pyruvate and α-ketoglutarate concentrations in response to ammonium chloride infusion in liver disease is compatible with a defect in intermediary metabolism (11). It is therefore relevant that Amatuzio, Shrifter, Stutzman, and Nesbitt (31) demonstrated accumulation of blood pyruvate in response to glucose infusion in hepatic coma, a finding which would be in accord with inhibition of final glucose oxidation by the high blood ammonia content in this condition. The delayed fall in pyruvate values found in control subjects receiving ammonium chloride could result from utilization of pyruvate in the tissues for transamination reactions.

Additional biochemical findings in hepatic coma compatible with deranged ammonia metabolism include high blood concentrations of glutamine (32), asparagine (33), and other amino acids, all of which may reflect increased amidation and transamination involved in disposal of ammonia. Precise details of the metabolic disorder still remain uncertain and our investigation failed to support or exclude the theory of cerebral α-ketoglutarate depletion (8). Augmentation rather than removal of peripheral blood α-ketoglutarate occurred in response to ammonium chloride administration in patients with liver disease and greatly increased amounts were available in the blood in hepatic coma. Nevertheless, the "blood-brain barrier" (34) may prevent replenishment of this keto-acid in the brain from peripheral blood (8). Examination of the spinal fluid revealed comparatively small quantities of α-ketoglutarate, which is compatible with the hypothesis that it passes from blood to spinal fluid with difficulty, but the higher concentrations present in hepatic coma fail to exclude its availability to the brain.

Certain practical points are emphasized by this investigation. It was confirmed that similar clinical and biochemical abnormalities occur in hepatic coma regardless of the etiology of the liver disease or nature of the precipitating factor (7). Blood ammonia estimations, particularly arterial concentrations, may be of diagnostic assistance in the fasting patient, but the close relationship postulated between arterial values, tissue uptake of ammonia and clinical status (9) was unreliable be-

cause blood ammonia concentrations reflect the amount of ammonia entering the circulation from the gastrointestinal tract and elsewhere, modified by release as well as uptake in various tissues, and the relationship between these factors is variable. The results of this investigation suggest the employment of antibiotics in treatment of hepatic coma (35). Glutamic acid also reduces blood ammonia values (36, 37) and its variable effect as a therapeutic agent may in part be due to relative impotence in the presence of a large influx of ammonia in the non-protein-deprived patient or the transient nature of its action in severe liver disease (38). On the other hand, its precursors, ammonia and α-ketoglutarate, were found in excess in peripheral blood in hepatic coma and elevated concentrations of glutamic acid itself have been reported in this condition (39). It appears from these considerations that assessment of any agent in the therapy of hepatic coma should be compared with the effect of protein deprivation and include estimations of arterial ammonia concentrations while also taking into account the importance of nitrogenous material in the gastrointestinal tract.

SUMMARY

1. In 27 patients studied in hepatic coma, blood ammonia concentrations were more frequently elevated in the artery than in the peripheral vein, but a good correlation with clinical status was evident at neither site. Fasting patients with uncomplicated liver disease had normal blood ammonia concentrations and the height of arterial values in hepatic coma was broadly related to the amount of nitrogenous material in the intestines. Values fell towards normal with protein withdrawal and broad spectrum antibiotic therapy, but a later elevation occurred in fatal cases, despite continuation of this regimen. The origin of this ammonia was uncertain and could only sometimes be attributed to release from brain or muscle.

2. Uptake of ammonia by peripheral tissues was impaired in liver disease relative to normal capacity. During the late stages of coma, poor uptake, equilibrium or release of ammonia by peripheral tissues or brain occurred, despite high arterial concentrations.

3. Elevation of blood pyruvate and α-ketoglutarate values paralleled the high blood ammonia concentration in hepatic coma. A rise in blood concentrations of these keto-acids followed the administration of ammonium chloride to patients with liver disease, but did not take place in control subjects. It is suggested that the high concentrations of keto-acids in hepatic coma represent a defect in intermediary metabolism due to impaired utilization of ammonia and faulty removal from the blood by a diseased liver. Concentrations of pyruvate in cerebro-spinal fluid were comparable to those in arterial blood, but relatively small amounts of α-ketoglutarate were found there, although values were above normal in hepatic coma.

4. The significance of blood ammonia concentrations in hepatic coma must take into account ammonia entering the system from the gastrointestinal tract, uptake or release of ammonia at various sites and the possibility of pre-existing defects in ammonia utilizing systems. Protein withdrawal with broad spectrum antibiotics effectively reduced blood ammonia values.

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THE INTERNAL DISTRIBUTION OF HYDROGEN IONS WITH VARYING DEGREES OF METABOLIC ACIDOSIS [1]

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When a mineral acid is administered to animal or man, a substantial fraction is buffered in sites other than blood or interstitial fluid. Van Slyke and Cullen (1) first observed that total blood volume could account for the buffering of only one-sixth of a mineral acid load and suggested that buffer substances throughout the body, including those of the tissues, are utilized in the defense against metabolic acidosis. Recent studies in several laboratories have provided more detailed quantitative data on the internal distribution of administered hydrogen ions. Swan and Pitts (2) in nephrectomized dogs and Schwartz, Jenson, and Relman (3) in normal human subjects have demonstrated that only about one-half of a mineral acid load is buffered in the extracellular space and red blood cells. The remaining hydrogen ions exchange with sodium and potassium from tissue and bone and are presumably buffered in these areas.

The purpose of this investigation was to determine whether the partition of administered hydrogen ions between the intra and extracellular phases is the same regardless of the magnitude of the acid load, or whether variation in the severity of the acidosis results in preferential utilization of one or the other of the buffer compartments. In the present experiments the distribution of hydrogen was determined in dogs made progressively acidotic by the intravenous administration of hydrochloric acid. The data demonstrate that when equilibrium is allowed to occur the partition of hydrogen ions between extracellular and intracellular buffers is essentially unaffected by the degree of acidosis.

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[2] This work was done during the tenure of an Established Investigatorship of the American Heart Association.

[3] Supported by a grant from the Royal Norwegian Ministry of Foreign Affairs and by a Fulbright Travel Grant.

[4] This work was done during the tenure of a Research Fellowship of the American Heart Association.

MATERIALS AND METHODS

Experiments were performed in 24 healthy mongrel dogs weighing from 10.5 to 21.8 Kg. Small doses of morphine were used for sedation during the preparatory manipulations. The femoral artery and vein were cannulated with polyvinyl catheters. An indwelling catheter was inserted in the bladder, and a Levine tube was introduced into the stomach and left in place for the duration of the experiment. Heparinized arterial blood was collected anerobically for determination of pH, total carbon dioxide content, sodium, potassium, chloride, hematocrit, hemoglobin and total protein. Measurements were made of urinary titratable acid, ammonium, phosphorus, sodium, potassium, and chloride. The volume of gastric juice was measured periodically, and a 5-cc. aliquot was removed for determination of total acid content. The remainder of the gastric content was promptly returned to the stomach. The analytic procedures employed in this study have been described in a previous paper from this laboratory (4). Following a control period of 30 to 60 minutes, during which urine and several blood samples were collected, hydrochloric acid in isotonic glucose was administered intravenously by means of a Bowman infusion pump. Employing concentrations of 0.5 to 1.2 N hydrochloric acid, it was ordinarily possible to limit the amount of fluid given to less than 5 per cent of estimated total body water. At the completion of the experiments, most of the animals were sacrificed and the volume and total acidity of the stomach contents determined. The small intestine was also regularly examined, but the volume of fluid was small and no measurement of volume or composition was attempted.

Two types of experiments were performed. In the first group of 14 *continuous infusion experiments* hydrochloric acid was administered without interruption until the animal died or the plasma bicarbonate concentration fell to less than 5 mEq. per L. In 11 experiments the rate of acid infusion was roughly 80 microequivalents per kilogram per minute and in three, 160 microequivalents per kilogram per minute. In the second group of ten *intermittent infusion experiments* hydrochloric acid was

TABLE I

Effects of continuous infusion of hydrochloric acid

Dog No. 98. Weight 14.1 Kg.; HCl 0.750 N in 5% glucose infused at approximately 80 microequivalents/Kg./min.; Hemoglobin 17.2 Gm./100 cc.; Hematocrit 41.5%; Plasma protein 5.8 Gm./100 cc.

A. Experimental observations

Total elapsed time	Plasma						Urine							Gastric juice	
	pH	pCO_2	HCO_3	Cl	Na	K	Flow	pH	NH_4	TA	Cl	Na	K	Volume	Total H
min.		*mm. Hg*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*cc./min.*		*μEq./min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*cc.*	*mEq.*
−39	7.37	43	24.2	111	146	3.5									
0	7.37	45	25.4	112	146	3.6	0.35	6.70	9	2	29	18	28	5	0
	HCl infusion begun at 0 min. at 1.35 cc./min.														
15	7.28	46	20.7												
30	7.26	41	17.7	118	144	3.6									
60	7.14	38	12.4	124	141	3.9	0.35		8	18	12	6	18	13	0
90	7.06	33	9.1	127	141	4.2									
120	6.94	30	6.3	130	140	5.2	0.63	5.34	22	12	65	26	34	23	0.1
150	6.81	22	3.4	134	138	6.5									
165	6.73	20	2.5												
180	6.68	17	2.0	134	133	8.4	0.37		8	59	47	22	24	0	0
201	Dog died. Gastric content at autopsy: 35 cc., containing 0.35 mEq. H^+.														

B. Derived data

Total elapsed time	ECF volume*	Hydrogen infused	Distribution of administered hydrogen ions							Shifts in IC electrolytes	
			Urine†	Gastric	ECF HCO_3	Plasma protein	RBC HCO_3	Hgb	ICF	ΔNa	ΔK
min.	*L.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*
30	2.91	30.3	0	0	20	0.4	1.6	2.7	5	−7	0
60	2.99	30.3	0	0	16	0.4	1.1	3.2	9	−3	−1
90	3.12	30.3	1	0	10	0.3	0.6	2.1	16	−19	−1
120	3.25	30.3	1	0	9	0.4	0.6	3.3	16	−15	−4
150	3.35	30.3	0	0	10	0.5	0.6	3.8	15	−9	−5
180	3.56	30.3	0	0	5	0.5	0.3	4.1	20	−11	−8

* Initial ECF volume estimated as 20 per cent BW (2.82 L.).

† Urine was collected less frequently than blood, and urine acid losses were assumed to be constant over the collection interval.

administered in an interrupted manner, each experiment consisting of a series of 45-minute infusions during which acid was given at the rate of approximately 80 microequivalents per kilogram per minute. A two-hour equilibration period was allowed after each of the infusion periods.[5] Blood specimens were obtained at the beginning and end of each acid infusion, and serially 60, 105, and 120 minutes after acid had been discontinued. In all experiments reported the cumulative loss of gastric juice was less than 100 cc. (10 mEq. or less of H^+) and urine increment in acid excretion (ammonium plus titratable acid) less than 10 per cent of the administered load. Several additional intermittent infusion experiments were excluded from the study because gastric or urinary losses considerably exceeded these limits.

[5] This interval was used because in preliminary studies it was found that the rise in plasma bicarbonate concentration, which began immediately after each infusion was stopped, was essentially complete in two hours.

Calculations

Hydrogen ion balance was calculated in a manner previously described (3). Retained acid was taken as the difference between hydrochloric acid administered and the increment in gastric acid secretion and urine acid excretion (ammonium plus titratable acid). The distribution of hydrogen ion in the buffers of the blood was estimated in the fashion described by Singer and Hastings (5). For these calculations blood volume was taken to be 70 cc. per kilogram of body weight, and red cell and plasma volume were considered to be constant, ignoring the small error introduced by withdrawal of blood. Initial extracellular fluid volume was taken to be 20 per cent of body weight and subsequent alterations estimated from changes in the "chloride space." The buffer contribution of extracellular bicarbonate was calculated from the observed reduction in plasma concentration with the use of a combined Donnan and plasma water correction factor of 1.11. Intracellular buffering was taken as the difference between total retained hydrogen ions and that

buffered by the extracellular space.[6] Internal exchanges of sodium and potassium were estimated in the usual fashion from "chloride space" calculations. Shifts of potassium were interpreted on the assumption that there were no significant changes in nitrogen balance in the course of the experiment. Hemolysis was seen frequently at bicarbonate levels of less than 6 mEq. per L., but its effect upon calculated potassium shifts was ignored since the potassium content of dog erythrocytes is only from 4 to 12 mEq. per L. (7).

RESULTS

Part I. Continuous infusion experiments

Distribution of hydrogen ions among the body buffers. Table IA summarizes the data from one of 14 experiments in which acid was infused continuously. The upper half of Figure 1 shows the plasma bicarbonate concentration plotted as a function of time in minutes. The rate of decrease in bicarbonate concentration was initially rapid but slowed progressively during the experiment. Thus, in the first half-hour a reduction of 8 mEq. per L. occurred but in the last half-hour of the study an equal quantity of infused acid reduced bicarbonate concentration by only 1.4 mEq. per L. Twelve of the 14 experiments demonstrated a similar pattern of change in plasma bicarbonate concentration.

Table IB gives the calculated values for distribution of acid based upon measurements made at regular intervals during the course of the acid infusion. Acid distribution between intracellular and extracellular fluid for each period of infusion is shown *non-cumulatively* in the lower half of Figure 1 and in Table IB, and demonstrates an apparent small intracellular buffer contribution early in the study with a much larger intracellular buffer contribution during the latter part of the experiment. Thus, extracellular buffers accounted for 83 per cent and intracellular buffers 17 per cent of the first 2.1 mEq. per kilogram of acid administered. In contrast, during the final period of the experiment the extracellular space buffered only 34 per cent of the last 2.1 mEq. per kilogram of acid administered, the intracellular phase now having buffered 66 per cent.

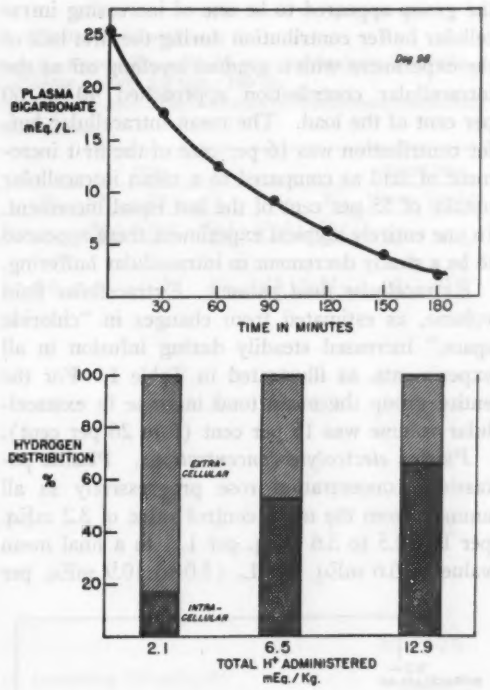

FIG. 1. THE EFFECTS OF CONTINUOUS INFUSION OF HYDROCHLORIC ACID

Plasma bicarbonate concentration is shown in the upper portion. Calculated hydrogen distribution is shown *non-cumulatively* in the lower portion. Note the exponential fall in plasma bicarbonate concentration and the apparent progressive increase in intracellular buffering as acidosis becomes more severe.

In the group of 14 continuous acid infusion experiments, the total acid administered varied from 6.8 to 15.8 mEq. per Kg., a quantity sufficient to reduce plasma bicarbonate concentration in each case to less than 5 mEq. per L. Figure 2 summarizes the calculations of intracellular buffering for the entire group of continuous infusion experiments. Intracellular buffering for the 14 experiments is plotted against total administered acid but in a *non-cumulative* fashion. Each point, therefore, represents only that buffering calculated to have occurred in the immediately preceding period of infusion. The overall pattern for

[6] Red blood cell buffers make a relatively small and constant contribution to total buffering and have been included with the buffers of the extracellular fluid. The term "intracellular" is used broadly in this discussion to refer to all non-extracellular areas in which hydrogen buffering might occur. This probably includes not only muscle and other soft tissues, but also bone, which has been demonstrated to have a large labile store of sodium and potassium (6).

the group appeared to be one of increasing intracellular buffer contribution during the first half of the experiment with a gradual levelling off as the intracellular contribution approached 40 to 60 per cent of the load. The mean intracellular buffer contribution was 16 per cent of the first increment of acid as compared to a mean intracellular uptake of 55 per cent of the last equal increment. In one entirely atypical experiment there appeared to be a steady decrement in intracellular buffering.

Extracellular fluid volume. Extracellular fluid volume, as estimated from changes in "chloride space," increased steadily during infusion in all experiments, as illustrated in Table I. For the entire group the mean total increase in extracellular volume was 15 per cent (6 to 26 per cent).

Plasma electrolyte concentrations. Plasma potassium concentration rose progressively in all animals from the mean control value of 3.2 mEq. per L. (2.5 to 3.6 mEq. per L.) to a final mean value of 6.6 mEq. per L. (5.0 to 10.9 mEq. per L.). The mean increase in concentration was 3.4 mEq. per L. (0.6 to 7.9 mEq. per L.).

Plasma sodium concentration decreased slightly but significantly during infusion from the mean control value of 144 mEq. per L. (139 to 151 mEq. per L.) to a final mean value of 138 mEq. per L. (133 to 145 mEq. per L.).

Exchanges of electrolytes. The estimated total shift of potassium from intracellular fluid for the group of experiments had a mean value of 1.33 (± 0.91) mEq. per Kg. The estimated total shift of sodium from intracellular fluid for the group as a whole had a mean value of 3.26 (± 1.67) mEq. per Kg. and comprised roughly two-thirds of the estimated intracellular cation loss.

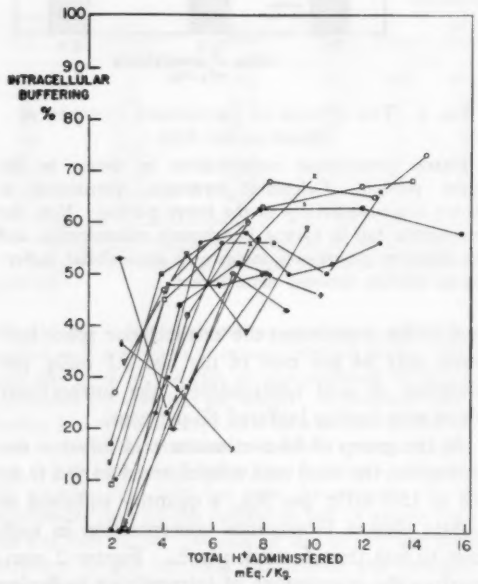

FIG. 2. APPARENT INTRACELLULAR BUFFER CONTRIBUTION DURING CONTINUOUS INFUSION OF HYDROCHLORIC ACID

Intracellular buffering for the 14 experiments is plotted against total administered acid but in a *non-cumulative* fashion. Each point, therefore, represents only that buffering calculated to have occurred in the immediately preceding period of infusion. Note the apparent rise in intracellular buffering as acidosis becomes more severe.

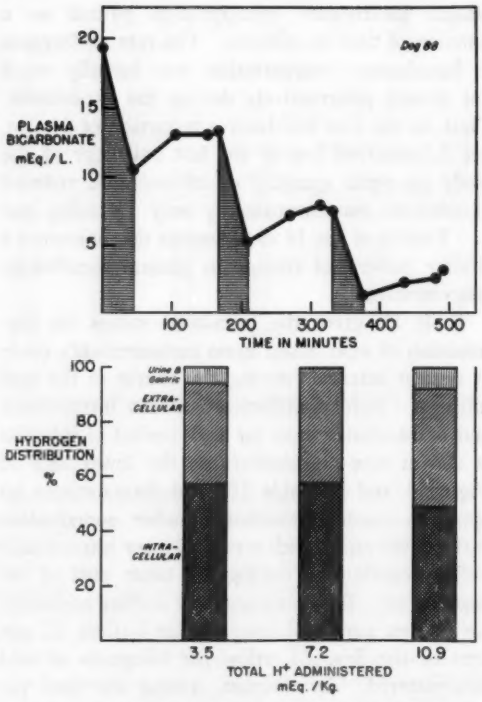

FIG. 3. THE EFFECTS OF INTERMITTENT INFUSION OF HYDROCHLORIC ACID

Plasma bicarbonate concentration is shown in the upper portion where the periods of infusion are indicated by the shaded areas. Calculated hydrogen ion distribution is shown *non-cumulatively* in the lower portion. Note the rebound in plasma bicarbonate concentration during the equilibration period following each infusion, and the roughly constant contributions of extracellular and intracellular buffers at all degrees of acidosis.

TABLE II

Effects of intermittent infusion of hydrochloric acid

Dog No. 88. Weight 21.8 Kg.; HCl 1.16 N in 5% glucose infused at approximately 80 microequivalents/Kg./min.; Hemoglobin 16.4 Gm./100 cc.; Hematocrit 52%; Plasma protein 6.2 Gm./100 cc.

A. Experimental observations

Total elapsed time	Plasma						Urine							Gastric juice	
	pH	pCO_2	HCO_3	Cl	Na	K	Flow	pH	NH_4	TA	Cl	Na	K	Volume	Total H
min.		*mm. Hg*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*cc./min.*		*μEq./min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*cc.*	*mEq.*
−101	7.39	33	19.1	112	147	3.0									
0	7.36	35	19.0	112	147	3.1	0.21	6.2	11	5	40	45	11	0	0
	HCl infused from 0–45 min. at 1.46 cc./min.														
45	7.23	26	10.5	122	144	3.6									
105	7.32	26	13.0												
150	7.37	23	13.0												
165	7.35	24	13.1	120	145	4.0	0.50	6.0	19	15	59	36	53	25	1.4
	HCl infused from 165–210 min. at 1.49 cc./min.														
210	7.10	17	5.2	132	143	4.6									
270	7.20	19	7.2												
315	7.25	18	7.7												
330	7.26	17	7.4	126	142	6.4	0.16		2	1	8	2	7	5	†
	HCl infused from 330–375 min. at 1.44 cc./min.														
375	6.87	7	1.2	138	140	7.2									
435	7.07	8	2.2												
480	7.06	9	2.4												
495	7.09	10	3.0	132	139	8.5	0							85	7.0
	Animal sacrificed. Gastric content at autopsy: 85 cc. containing 7.0 mEq.H^+														

B. Derived data

Infusion period	ECF volume*	Hydrogen infused	Distribution of administered hydrogen ions							Shifts in IC electrolytes	
			Urine	Gastric	ECF HCO_3	Plasma protein	RBC HCO_3	Hgb	ICF	ΔNa	ΔK
	L.	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*
I	4.62	77	3	1.4	25	0.1	2.4	1.2	44	−29	−12
II	4.95	78	0	0	27	0.4	2.2	3.2	45	−34	−13
III	5.22	76	0	5.6	23	0.8	1.8	6.8	38	−22	−13

* Initial ECF volume estimated as 20 per cent BW (4.36 L.).
† Assumed to have been same as period I.

pH and pCO_2. The mean control pH was 7.35 (7.28 to 7.45). pH regularly fell during acid administration in a roughly linear fashion. The mean final pH was 6.74 (6.45 to 7.05). pCO_2 regularly fell in a roughly linear fashion during acid administration from a mean initial value of 42.4 mm. Hg (32 to 49 mm. Hg) to a final mean value of 17 mm. Hg (9 to 24 mm. Hg).

Part II. Intermittent infusion experiments

Distribution of hydrogen ions among the body buffers. Table IIA and Figure 3 summarize data from one of ten experiments in which a two-hour equilibration period was permitted after each of a series of 45-minute acid infusions. In the upper half of Figure 3 the periods of acid infusion are represented by the shaded areas. Plasma bicarbonate concentration fell sharply during each period of acid administration, rose sharply during the first hour of equilibration, and gradually levelled off by the end of two hours. In this experiment bicarbonate concentration rose during equilibration by an amount equal to approximately 30 per cent of the initial fall. The bicarbonate decrements resulting from the first two acid infusions were of the same order of magnitude, but in the last period it was slightly less. In each experiment the pattern of change in plasma bicarbonate concentration was essentially the same,

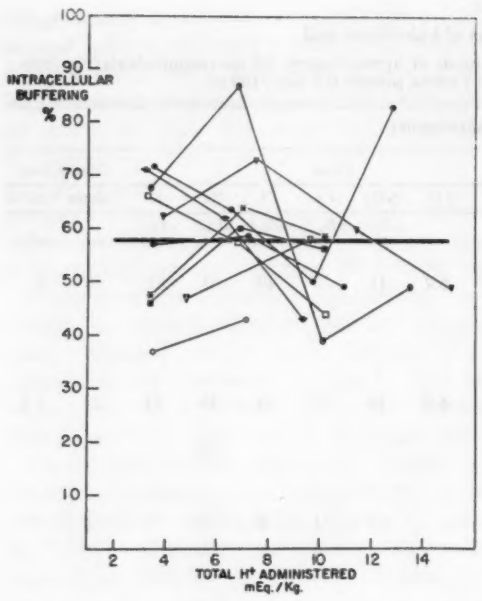

FIG. 4. INTRACELLULAR BUFFERING OF SUCCESSIVE ACID INCREMENTS DURING INTERMITTENT HYDROCHLORIC ACID INFUSION EXPERIMENTS

Intracellular buffering for the ten experiments is plotted against total administered acid but in a *non-cumulative* fashion. Each point, therefore, represents only that buffering calculated to have occurred at equilibrium following the immediately preceding period of infusion. Note the absence of any consistent trend in intracellular buffering as acidosis becomes more severe, and the random variations about the mean value. Mean of average slopes $= -0.507$ (± 2.37), not significantly different from zero ($t = 0.642$, $p > 0.40$). Mean intracellular buffering = 57.6 per cent (standard deviation ± 11.6, standard error ± 2.08).

showing a sharp fall during acid infusion and a rapid rise during the first hour of equilibration. During the second hour there was usually only a small additional rise. For the group as a whole, taking the mean of all the periods, the reduction in bicarbonate concentration during each infusion was 8.0 mEq. per L. During equilibration the rise in bicarbonate concentration in most instances tended to be slightly smaller in the second and third periods than in the first period of acid administration. The mean values for the net decrement in bicarbonate concentration after equilibration for successive periods were 6.3, 4.6, and 4.6 mEq. per L., respectively.

Table IIB gives the calculated values for distribution of acid based upon measurements made two hours after each infusion. Acid distribution between intracellular and extracellular fluid for each infusion period is shown *non-cumulatively* in the lower half of Figure 3. Intracellular buffering varied between 50 and 58 per cent, and extracellular buffering between 37 and 43 per cent during the course of the experiment. The remaining fraction of administered hydrogen ions appeared in urine and gastric juice.

In the entire group of intermittent infusion experiments, the total acid administered varied from 7.1 to 15.1 mEq. per Kg. Some animals tolerated only two periods of infusion before developing lethal acidosis, others three and four. As will be seen, the amount of acid administered did not affect its pattern of distribution. Figure 4 summarizes the calculations of intracellular buffering for the entire group of intermittent experiments. Intracellular buffering for the ten experiments is plotted against total acid administered but in a *non-cumulative* fashion. Each point, therefore, represents only that buffering calculated to have occurred in the immediately preceding period of infusion. The slope for the best-fitting straight line was calculated for each of the ten experiments. The mean of the slopes of these lines was -0.507 (± 2.37), a value not significantly different from zero ($t = 0.642$, $p > 0.40$). The mean percentage buffering by intracellular fluid was 57.6 per cent (standard deviation ± 11.6, standard error ± 2.08).

The utilization of extracellular bicarbonate in the group as a whole did not appear to differ significantly with successive periods of infusion. For each experiment the average slope was calculated for a line defining extracellular bicarbonate utilization as a function of total administered acid. The mean of the ten average slopes was -0.411 (± 1.15), a value not significantly different from zero ($t = 1.07$, $p > 0.30$). The mean buffer contribution of red cells and plasma protein for the 31 infusion periods was 7.1 ± 3.7 per cent of the administered load.

Extracellular fluid volume. Extracellular fluid volume, as estimated from changes in "chloride space," increased steadily from period to period in all experiments, as illustrated in Table II. For the entire group the mean total increase in ex-

tracellular volume was 17 per cent (10 to 28 per cent).

Plasma electrolyte concentrations. Plasma potassium concentration rose progressively in all animals from the mean control value of 2.9 mEq. per L. (2.4 to 3.1 mEq. per L.) to a final mean value of 6.7 mEq. per L. (5.5 to 8.5 mEq. per L.). The mean increase in concentration was 3.8 mEq. per L. (2.9 to 5.4 mEq. per L.).

Plasma sodium concentration decreased slightly but significantly during infusion from the mean control values of 144 mEq. per L. (139 to 150 mEq. per L.) to a final mean value of 137 mEq. per L. (131 to 142 mEq. per L.).

Exchanges of electrolytes. The estimated shift of potassium from the intracellular space had a mean value of 2.58 (± 1.16) mEq. per Kg. Except for a consistently smaller shift with the first infusion, intracellular potassium loss was roughly constant for additional equal increments of acid. This is well illustrated by data shown in Table II.

Renal excretion of potassium increased in all but the three experiments in which oliguria developed. In the seven experiments where urine volume remained at the control level or increased, the total potassium loss estimated from the increment above control excretion averaged 2.3 mEq. per Kg. This urinary loss represented 68 per cent of the potassium calculated to have been displaced from cells.

The mean intracellular sodium loss for the group as a whole was 3.81 (± 1.61) mEq. per Kg., and comprised roughly 60 per cent of the estimated intracellular cation loss.

For the group as a whole the intracellular cation loss was of the same order of magnitude in each successive infusion period. For each experiment the average slope was calculated for a line defining intracellular cation loss as a function of total administered acid. The mean of the average slopes was 1.34 (± 2.70), a value not significantly different from zero ($t = 1.40$, $p > 0.19$). The mean value for hydrogen calculated to have entered cells in this group of experiments was 6.33 (± 1.86) mEq. per Kg., and the mean intracellular cation loss was 6.39 (± 2.56) mEq. per Kg. There is no significant difference between these values ($t = 0.05$, $p > 0.90$).

pH and pCO_2. The mean control pH was 7.33 (7.26 to 7.38). For the group as a whole there was a roughly linear fall in pH with successive infusion periods to a final mean value of 7.03 (6.81 to 7.15). At equilibrium after six of 31 infusion periods, pH returned to within 0.02 units of the pre-infusion value.

The mean control pCO_2 was 39 mm. Hg (34 to 45 mm. Hg). For the group as a whole there was a roughly linear fall in pCO_2 with successive infusion periods to a final mean value of 20 mm. Hg (9 to 31 mm. Hg). In seven of 31 infusion periods, however, there was no significant change in pCO_2 (± 3 mm. Hg).

DISCUSSION

The present studies indicate that the distribution of hydrogen ions among the body buffers is not affected by the magnitude of the acid load. The apparent predominance of extracellular buffering early in the course of continuous acid infusion does not represent the "steady state" response to metabolic acidosis. The fact that bicarbonate concentration rises sharply when the acid infusion is stopped reveals that the curvilinear fall in bicarbonate concentration during continuous acid infusion is the resultant of two opposing processes. On the one hand, the infusion of acid converts bicarbonate to carbonic acid thus tending to lower plasma bicarbonate concentration. On the other hand, the diffusion of bicarbonate from the interstitial fluid to plasma and the transfer of hydrogen ions from interstitial fluid to the intracellular space both tend to elevate plasma bicarbonate concentration. In the continuous infusion experiments this process did not have a chance to approach equilibrium until the late stages of the experiments when the bulk of administered acid had been given adequate time for distribution. In the intermittent infusion experiments, where equilibrium was allowed to occur at successive points during the study, the partition of hydrogen ions between extracellular and intracellular buffers was found to be essentially unaffected by the degree of acidosis.

Within individual experiments of the intermittent group there were often rather wide variations from period to period in the estimated contributions of the two buffer compartments, the standard deviation from the mean in Figure 4 being ± 11.6 per cent. It seems probable that the most

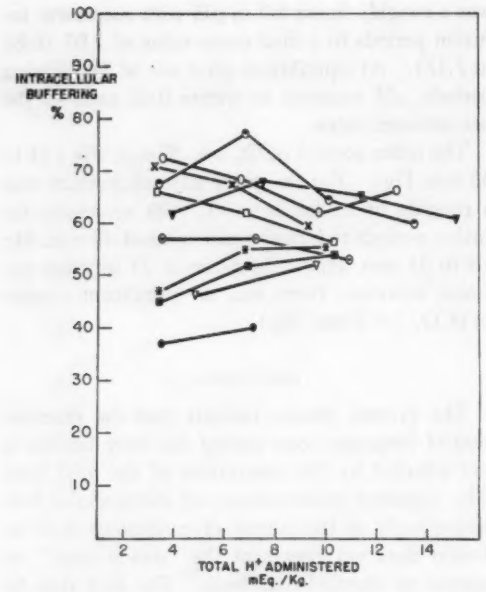

FIG. 5. INTRACELLULAR BUFFERING OF TOTAL ADMINISTERED ACID DURING INTERMITTENT HYDROCHLORIC ACID INFUSION EXPERIMENTS

Each point represents the *total* intracellular buffer contribution up to the time of any given equilibrium observation. Note that the variation in buffering of the total load by the intracellular area is less than 10 per cent in the course of nine of the ten experiments.

important factor in the production of these variations was the secretion and absorption of gastrointestinal juices. The single most critical measurement upon which the calculation of hydrogen distribution depends is plasma bicarbonate concentration. Sudden transfer of acid between plasma and the gastrointestinal tract by either secretion or reabsorption of gastric juice would markedly influence plasma bicarbonate concentration until equilibrium between plasma, interstitial fluid and tissues had been achieved. If such exchanges occurred shortly before collection of the "equilibrium" blood, significant distortion of the calculations of buffer distribution would result. Although total gastric losses were modest in the present series of experiments, changes in gastric acid content from period to period were probably large enough to have introduced significant artifacts and were probably the chief cause of the random variations in calculated buffer distribution shown in Figure 4. On the other hand, urinary acid losses were constant enough presumably to have been of lesser significance.

Errors in calculation of extracellular fluid volume are probably of minor consequence in the interpretation of these experiments. As estimated from "chloride space," extracellular volume increased steadily in the course of every experiment. The mean increase in extracellular fluid agreed closely with the change in inulin and radiosulfate space found by Swan and Pitts following a single acid infusion in nephrectomized dogs (2). Although an assumed figure of 20 per cent of body weight was used for initial extracellular volume, substitution of other values between 15 per cent and 30 per cent does not significantly alter the calculated pattern of acid distribution.

The sources of error considered above are probably of the same absolute magnitude regardless of the quantity of acid administered. For this reason their relative importance in affecting the estimate of total hydrogen distribution based on a single measurement of plasma composition will be progressively less as acidosis increases. Thus when we consider the distribution of the *total* acid load administered up to any given time of observation (Figure 5) rather than the distribution of only the small increments of acid added within each period, the variation within each experiment is much smaller than that shown in Figure 4. In nine of the ten experiments the variation in intracellular buffering was less than 10 per cent. In metabolic acidosis the plasma bicarbonate concentration is affected by the total load of acid buffered in the extracellular space up to the time of blood sampling. Figure 5 suggests that at equilibrium in any given subject the quantity of acid buffered extracellularly bears a reasonably close relationship to the acid buffered within the cells. It follows, therefore, that a single bicarbonate determination will give a satisfactory though approximate estimate of the per cent reduction in total body buffer reserves. The rather large differences in distribution of acid between experiments (Figure 5) does not affect this conclusion, but it does suggest the difficulty of estimating the quantity of acid responsible for producing a given reduction in plasma bicarbonate concentration. The differences in the relative distribution of acid from subject to subject may in part have been the result

of variations in the size of buffer stores, depending for example on the relative amounts of fat and lean tissue in a given animal.

In the present study hyperkalemia was observed in every experiment despite, in most instances, a normal urine flow and a marked increase in potassium excretion. Previous workers have noted sizable potassium shifts to extracellular fluid when metabolic acidosis was induced in nephrectomized animals (2, 8). The present data suggest that in acute metabolic acidosis, even with intact kidneys, the rate at which potassium is displaced by hydrogen exceeds the kidney's excretory capacity for this ion. The degree of hyperkalemia was directly related to the severity of the acidosis, although the rise in plasma potassium concentration was generally smallest in the animals with the largest increments in potassium excretion. Potassium concentration rose by an average of 3 to 4 mEq. per liter, but it is probable that elevations of this degree would not be maintained in chronic acidosis where more complete renal compensation could occur. It should be noted, however, that serum potassium concentration tends to be maintained at slightly elevated levels relative to total body potassium stores as long as pH remains low (9).

The mechanism governing the distribution of hydrogen ions through total body buffer stores is not clear from the present experiments. It seems likely that hydrogen ion distributes itself across cell membranes according to its electrochemical gradient (10). Thus any change in intracellular-extracellular gradient of hydrogen would tend to produce a shift of this ion. The present data show a progressive reduction in extracellular pH as more acid was administered, and it seems probable that this increase in hydrogen ion concentration was the major factor in the transfer of hydrogen ions to the intracellular space. There were, however, exceptions to this pattern in individual experiments where despite a fall in plasma bicarbonate concentration and a sizable movement of hydrogen into cells, there was little or no change in extracellular pH. Nevertheless, hydrogen transfer may still be explained in terms of a change in hydrogen gradient, since in these instances extracellular pH was preserved by virtue of a sharp reduction in pCO_2. Since a fall in pCO_2 would probably be accompanied by a rise in intracellular pH, even in the presence of a constant extracellular pH the gradient would be altered in a way favoring the inward movement of hydrogen ions. Thus, although there is no entirely predictable pattern in the change of either pH or pCO_2, their individual effects on hydrogen gradient may act together to produce roughly the same hydrogen distribution. Further studies with control of pCO_2 may serve to clarify this problem.

SUMMARY

Hydrochloric acid was administered intravenously to dogs in order to define the internal distribution of administered hydrogen ions with varying degrees of metabolic acidosis. Experiments in which acid was infused continuously appeared to indicate preferential utilization of extracellular buffers in the initial phase with the contribution of intracellular buffers becoming more important as the acidosis increased in severity. However, when the acid load was administered intermittently, allowing time for equilibrium to occur, the partition of hydrogen ions between extracellular and intracellular buffers was found to be essentially unaffected by the degree of acidosis. The data indicate that at equilibrium the percentage reduction in plasma bicarbonate concentration provided an approximate index of the percentage reduction in total body buffer stores.

ACKNOWLEDGMENT

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ANNOUNCEMENT OF MEETING

The 49th Annual Meeting of the American Society for Clinical Investigation will be held in Atlantic City, New Jersey, on Monday, May 6, 1957, with headquarters at the Chalfonte-Haddon Hall. The scientific session will begin at 9 A.M. at the Steel Pier Theater.

A STUDY OF THE MECHANISM OF SECRETION OF THE SODIUM-RETAINING HORMONE (ALDOSTERONE) [1, 2]

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Aldosterone, which has been isolated from the adrenal cortex and chemically characterized, is the most powerful sodium-retaining hormone among the known naturally occurring adrenal steroids (1). As such, it seems probable that it plays a dominant part in the regulation of sodium and potassium metabolism.

There is considerable evidence that aldosterone may be unique among adrenal steroids in that its rate of secretion is largely independent of anterior pituitary control. This is suggested by the absence of atrophy of the zona glomerulosa in rats and dogs after hypophysectomy (2, 3), by the persistence of aldosterone in the adrenal venous blood of hypophysectomized dogs (4) and by the observation that some patients with acute pituitary insufficiency induced by total hypophysectomy conserve sodium normally (5) and excrete normal amounts of salt-retaining hormone in the urine (6).

In 1950, Luetscher, Neher, Wettstein, and Curtis (7, 8) first reported increased sodium-retaining activity in the urine of patients forming edema. In subsequent studies this group has identified the active substance in the urine as aldosterone. In addition, Luetscher and Axelrod (9) have reported increases in the excretion of hormone in normal subjects in response to short periods of sodium deprivation. The increase occurs without apparent alteration in the serum sodium concentration.

The present study was designed to examine the effects of changes in the dietary intake and of associated changes in the serum concentration of sodium and potassium ions upon the urinary excretion of the salt-retaining hormone. Certain aspects of the relationship between the distribution of sodium and potassium ions have been utilized to produce alterations of serum K and Na. Earlier studies have demonstrated that the administration of potassium to sodium-depleted dogs and human subjects produces a marked and sustained hyperkalemia which is not observed in subjects in normal electrolyte balance (10, 11).

The present report demonstrates that potassium ingestion, when accompanied by a rise in the serum potassium, may be associated with a pronounced increase of sodium-retaining activity in the urine. A fall in the serum sodium concentration *per se* does not appear to produce a comparable effect.

METHODS

The experiments were carried out on trained, unanesthetized mongrel dogs housed in metabolism cages. The animals were fed a synthetic diet of fixed electrolyte content containing casein, lard, dextrin, dextrose, vitamin and mineral supplements and agar-agar to provide approximately 80 calories per kilogram per day. The basal diet contained less than 1.8 mEq. of Na and less than 0.44 mEq. of K per day. When KCl was given it was administered either as a 20 per cent solution by stomach tube or incorporated into the diet. Urine was collected daily and pooled in 48-hour lots. After removal of 10-ml. aliquots for estimation of Na, K and Cl, the urines were stored at $-76°$ C. Stool specimens were not saved for analysis, but none of the dogs had diarrhea at any time. Methods for the estimation of serum and urinary sodium and potassium and chloride have been described previously (10).

For estimation of sodium-retaining activity, the thawed urine specimens were adjusted to pH 1 with 6 N HCl and extracted continuously for 24 hours with 350 ml. of redistilled methylene chloride in a Wolfe-Hirschberg extractor. The methylene chloride extract was washed successively five times with 50-ml. portions of 0.1 N NaOH and five times with 50 ml. of distilled water and taken almost to dryness at reduced pressure in an atmosphere of nitrogen. The extracts were dried further over P_2O_5 in a vacuum desiccator at room temperature and then stored at $-4°$ C. At the time of assay the dried

[1] This paper was presented at the 47th Annual Meeting of the American Society for Clinical Investigation, May 2, 1955.

[2] This work was supported in part by a grant from the U. S. Public Health Service, National Heart Institute (USPHS H-1275).

extract was first dissolved in 4 ml. of 50 per cent ethanol and dilutions of 1:16 and 1:80 were made.

The bioassay was carried out on male rats, adrenalectomized 24 hours before the test. After adrenalectomy the rats were offered distilled water ad libitum and a low sodium diet (Na 0.001 per cent, K 0.3 per cent). At the start of the assay 5 ml. of normal saline were injected intraperitoneally, and the bladder was emptied by electric shock. The animals were injected subcutaneously with either desoxycorticosterone acetate (DOCA) standards or unknowns in 0.25 ml. of 30 per cent ethanol, and placed in groups of three rats in metabolism cages. Duplicate groups of three rats were used for each DOCA standard (2, 10 and 50 micrograms) and for each unknown sample in 1:16 and 1:80 dilution. Human urine samples were handled similarly except that 24-hour collections were used, and extracts were assayed at 1:16, 1:80 and 1:400 dilutions. When samples were strongly active at all dilutions throughout the range tested, appropriate additional five-fold dilutions were employed until a suitable endpoint could be obtained. Further dilutions of inactive samples were tested since, with excessive amounts of aldosterone (near or greater than 10 micrograms per rat), sodium retention may not occur with this procedure.

At the end of a five-hour collection period the rats were forced to void by electric shock, the total urine volume was measured (4 to 10 ml. per group), and the amount of sodium excreted determined by flame photometry. The amounts of sodium excreted by the groups injected with DOCA were plotted on an arithmetic scale against the logarithm of the dose of DOCA (2, 10, 50 μg.). The standard curve thus obtained serves for estimation of the sodium-retaining equivalents of the unknowns. In this assay, 100 μg. of DOCA and 1.0 μg. of aldosterone have approximately equivalent activities. Using three groups of 3 rats each, the lambda is 0.23 (Lambda = standard deviation ÷ slope). Compounds B and F, in doses up to 1,500 μg., do not cause sodium retention nor interfere with the sodium-retaining activity of DOCA or aldosterone in this assay. In our hands, normal humans on unselected diet excrete 1 to 4 micrograms per 24 hours.

Active urine extracts were chromatographed on paper (12). The region of aldosterone was eluted and reassayed. With the larger amounts present in human urine, activity has been consistently confirmed in the eluate, and fluorescence in ultraviolet light and reduction with blue tetrazolium also gave results consistent with aldosterone. The active, crude extracts of urine consistently have been found to possess activity indistinguishable from pure aldosterone. Other groups have reported similar results (13).

Figure 1 illustrates the effect of orally administered KCl on the concentration of serum potassium in normal and sodium-depleted dogs. In four control dogs receiving 2 gm. of NaCl daily, the serum K was not appreciably

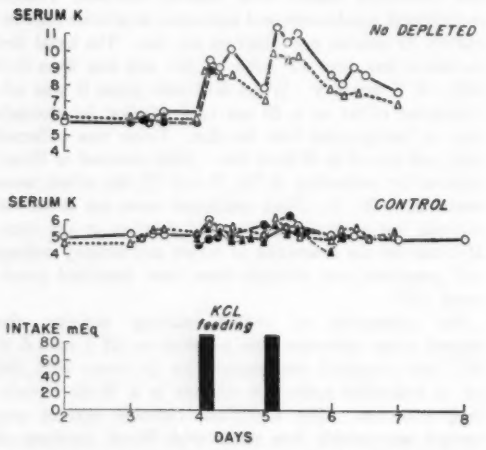

FIG. 1. HYPERKALEMIA RELATED TO SODIUM DEPLETION

Dogs rendered hyponatremic by peritoneal dialysis develop sustained hyperkalemia after feeding KCl. The same dose of KCl does not increase the serum potassium of animals not sodium depleted.

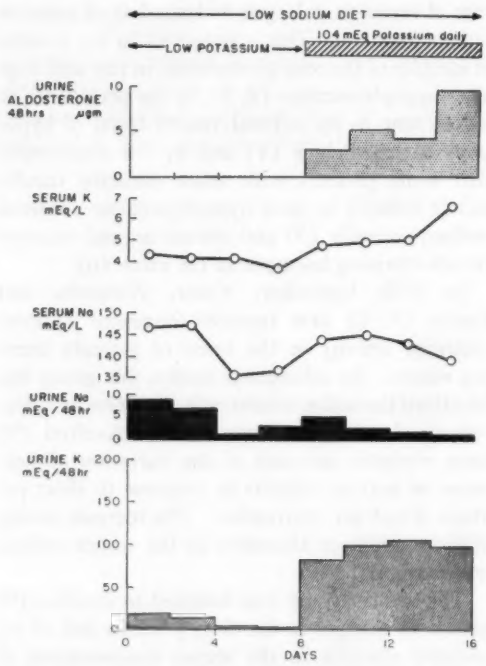

FIG. 2. EFFECT OF LOW AND HIGH POTASSIUM INTAKE UPON ALDOSTERONE, SODIUM AND POTASSIUM EXCRETION IN DOG N ON A LOW SODIUM DIET

No detectable sodium-retaining activity of urinary extracts was observed with a diet virtually free of sodium and potassium. When dietary KCl supplements were given, hyperkalemia developed and aldosterone-like activity appeared in the urine. No concurrent change in Na balance or serum Na was observed.

TABLE I

Feeding experiment in normal dogs

Dog	Experimental period	Consecutive days of balance period*	Daily intake Na (mEq.)	Daily intake K (mEq.)	Daily intake H_2O† (ml.)	Observed serum‡ Na (mEq./L.)	Observed serum‡ K (mEq./L.)	Aldosterone activity 48 hr. (Est. μg.)	Average daily urinary excretion§ Na (mEq.)	Average daily urinary excretion§ K (mEq.)	Average daily urinary excretion§ Vol. (ml.)
N	Control diet	7, 8	51	104	450	147	4.2	0	44	94	400
	Na and K free diet	1, 2	0	0	450	147	4.3	0	8.5	16.2	550
		3, 4	↓	↓	↓	148	4.2	0	6.1	10.6	470
		5, 6	↓	↓	↓	136	4.2	0	1.8	3.0	480
		7, 8	↓	↓	↓	137	3.8	0	2.2	1.8	525
	KCl added daily from day 9	9, 10	0	104	450	143	4.9	2.8	4.7	82.0	405
		11, 12	↓	↓	↓	145	5.1	5.2	2.1	98.0	415
		13, 14	↓	↓	↓	142	5.3	4.6	1.7	103.0	425
		15, 16	↓	↓	↓	139	6.7	9.0	1.2	92.0	410
W	Control diet (KCl added) on day 7, 8	5, 6	51	80	450	146	4.4	0	40	77	485
		7, 8	51	230	450	151	4.9	0	37	221	590
	Na free	1, 2	0	67	450	143	4.8	0	18	74	555
		3, 4	↓	67	↓	145	4.5	0	5.1	54	380
		5, 6	↓	104	↓	147	4.7	0.5	4.4	113	367
		7, 8	↓	104	↓	148	5.8	0.7	3.6	86	375
	Na and K free diet	9, 10	↓	0	↓	145	4.6	0	0.5	16.8	510
		11, 12	↓	↓	↓	144	3.6	0	0.4	2.4	455
		13, 14	↓	↓	↓	144	4.3	0	1.8	0.9	480
		15, 16	↓	↓	↓	143	3.8		0.5	0.7	478

* Refers to days from beginning of the particular diet.
† Distilled water given daily by stomach tube throughout.
‡ Refers to serum levels obtained in fasting state at end of the corresponding 48-hour balance period.
§ The average of two consecutive 24-hour measurements.

increased by KCl feeding. In contrast, dogs previously depleted of Na by peritoneal dialysis, so that the serum sodium was reduced to about 120 mEq. per L., uniformly developed a marked hyperkalemia when given the same amounts of KCl. Two dogs (M and R) died after receiving a single oral dose of 10 gm. of KCl when severely depleted of sodium. These dogs had tolerated even higher doses of KCl when on normal sodium intakes. This phenomenon has been utilized as a means of producing relatively sustained hyperkalemia.

The present studies were performed on 1) normal dogs, 2) dogs with diabetes insipidus, and 3) a single human subject with chronic congestive heart failure and edema.

1) Normal dogs

Three types of experiments were performed on these animals:

(*a*) *Feeding experiment* (*Table I, Figure 2*). One of two dogs (N) received a constant intake of a sodium and potassium-free diet for 8 days, and in the succeeding 8-day period KCl was added. In the other dog (W) this procedure was reversed, KCl being added in the first and absent in the second experimental period. Distilled water (450 ml.) was given daily by stomach tube.

(*b*) *Depletion experiment* (*Table II, Figure 3*). The dogs were first depleted of sodium chloride by peritoneal dialysis, according to the method of Darrow and Yannet (14), and then maintained on a constant diet free of sodium. Repeated dialysis was necessary in several instances to achieve significant hyponatremia. Dogs R and M received 18 mEq. of KCl on this diet whereas dogs W and E were kept on a K free as well as a Na free regimen. Distilled water (400 to 500 ml.) was given daily and, after a control period, KCl was added for two successive days by stomach tube.

(*c*) *Dilution experiment* (*Table III*). The serum sodium and potassium were reduced by a combination of forced hydration (1000 to 1500 ml. water by tube daily) and 2.5 units of Pitressin Tannate in Oil® injected twice daily. Sustained hemodilution and hypervolemia can be achieved in this way with reduction in serum sodium and potassium (15).

2) Dogs with diabetes insipidus (*D.I.*) (*Table IV*)

Diabetes insipidus was produced by electrocoagulation of the hypothalamic tracts by Dr. R. C. deBodo of the Department of Pharmacology, New York University College of Medicine. These dogs excreted from two to six liters of urine per 24 hours when having free access to food and water. They were studied during three successive periods: 1) unselected or stock diet, 2) sodium and potassium-free diet, and 3) with the addition of KCl

TABLE II

NaCl depletion experiments in normal dogs

Dog	Experimental period	Consecutive days of balance period	Daily intake Na (mEq.)	Daily intake K* (mEq.)	Daily intake H_2O† (ml.)	Observed serum Na (mEq./L.)	Observed serum K (mEq./L.)	Aldosterone activity 48 hr. (Est. μg.)	Average daily urinary excretion Na (mEq.)	Average daily urinary excretion K (mEq.)	Average daily urinary excretion H_2O (ml.)
R	Control diet	3, 4	51	107	500	148	4.0	0	46	101	525
	Balance study started 8 days after a dialysis	1, 2	0	18	500	128	4.2	1.5	0.5	8.8	460
		3, 4	↓	105	↓	137	6.9	11.2	2.3	85.0	560
		5, 6	↓	18	↓	130	6.2	16.8	1.3	27.5	435
		7, 8	↓	18	↓	129	4.5	3.6	0.7	14.2	490
	A second study started 6 days after another dialysis	1, 2	0	18	500	139	5.1	6.8	0.3	11.7	482
		3, 4	↓	105	↓	141	7.1	11.4	1.6	99.5	570
		5, 6	↓	18	↓	138	5.9	4.8	0.5	19.1	480
		7, 8	↓	18	↓	137	5.0	2.0	0.4+	13.5+	415+
		9, 10	↓	18	↓	137	4.8	0.8	0.4	11.7	445
M	Study started 7 days after dialysis	1, 2	0	18	400	134	5.8	5.1	0.4	15.4	265
		3, 4	↓	132	↓	131	6.8	9.6	2.2	107.0	355
		5, 6	↓	18	↓	129	5.0	5.0	0.4	27.0	322
W	Stock diet	1, 2			500	143	4.5	0	105	62	550
	Study started the day after a dialysis Na and K free diet	1, 2	0	0	500				0.5	15.1	325
		3, 4	↓	↓	↓	134	4.5	0.5	0.3	4.5	410
		5, 6	↓	↓	↓	133	4.3	0	0.3	2.9	480
		7, 8	↓	↓	↓	132	4.4	0.8	0.3	2.1	462
		9, 10	↓	94	↓	132	5.5	0.5	6.5	86.6	462
		11, 12	↓	0	↓	133	4.7	0.5	0.2	9.4	540
C	Stock diet	1, 2			500	145	4.2	0	42.4	46.3	412
	Balance study started 1 day after a dialysis	1, 2	0	0	500				0.8	27.4	490
		3, 4	↓	↓	↓	123	4.6	1.1	0.5	20.0	455
		5, 6	↓	↓	↓	125	5.2		0.7	14.0	525
		7, 8	↓	↓	↓	124	5.3	5.9	0.8	22.4	525
		9, 10	↓	67	↓	135	6.0	9.6	3.3	56.4	530
		11, 12	↓	0	↓	127	5.4	2.2	2.6	15.4	495

* K was given as KCl by stomach tube as a 20 per cent solution. The figures given are the averages of two doses for each two-day balance period.

† Represents total daily free water intake and does not include water used in mixing diet batches.

in amounts similar to those used in the normal dogs. Distilled water was allowed ad libitum in all periods.

3) A patient (Figure 5) with rheumatic heart disease

The patient was admitted to the metabolism ward and studied while on a constant regimen. He was a 52-year-old man with mitral insufficiency, auricular fibrillation and chronic, right-sided congestive failure. He had taken digitalis preparations daily for fifteen years and Mercuhydrin® injections, as often as twice a week, for over ten years. He had resorted to a low-sodium diet for at least ten years. Maintenance digitoxin was continued in the hospital. The sodium intake was kept constant throughout the study (12 mEq. daily). The rates of aldosterone excretion on a low (16 mEq. per day) and relatively high (140 mEq. per day) potassium intake were compared.

RESULTS

The results of the animal studies are summarized in the accompanying tables and in Figures 1–4.

Feeding experiments

In the experiments with two normal dogs (N and W, Table I) and two dogs with diabetes insipidus (T and E, Table IV), it was not possible to detect any sodium-retaining activity in the urine during periods of stock diet intake, or when KCl was given without simultaneous sodium deprivation. The KCl supplements ranged from 67 to 230 mEq. per day.

Dog N was kept on a diet free of sodium and potassium for a period of 8 days. During this

TABLE III

Dilution experiments in normal dogs

Dog	Experimental period	Consecutive days of balance period	Daily intake Na (mEq.)	Daily intake K (mEq.)	Daily intake H_2O* (ml.)	Observed serum Na (mEq./L.)	Observed serum K (mEq./L.)	Aldosterone activity 48 hr. (Est. μg.)	Average daily urinary excretion Na (mEq.)	Average daily urinary excretion K (mEq.)	Average daily urinary excretion H_2O (ml.)
N	Pitressin® 2.5 units b.i.d.	3, 4	0	0	ad lib.	142	4.4		5.0	4.8	380
	on days 5, 6, 7, 8	5, 6	0	0	1,125	122	3.6	0	8.1	3.5	615
		7, 8	0	204†	900	129	6.2	2.6	24.5	190.9	578
W	Pitressin in Oil® 2.5 units	3, 4	0	0	ad lib.	143	4.5	0	5.9	10.7	390
	b.i.d. on days 5, 6	5, 6	0	0	1,225	128	3.6	0	10.9	11.6	460

* Distilled water given by stomach tube daily or ad libitum as indicated.
† Dog vomited 200 ml., and this amount of K was subtracted from balance.

time, no sodium-retaining activity was detected in the urinary extracts. When 104 mEq. of potassium were added to the diet, significant hyperkalemia developed and salt-retaining activity appeared in appreciable amounts in the urine (Figure 2).

In a second normal dog (W), the administration of KCl did not produce as great an elevation of serum potassium, and insignificant amounts of sodium-retaining activity were detected in the urinary extracts.

Table IV presents data from similar feeding experiments in two dogs with diabetes insipidus. These animals also, as stated above, appear to excrete little or no aldosterone-like material when on a stock diet or on a diet free of sodium and potassium. When potassium chloride was added to the diet, an increase in the sodium-retaining activity of the urinary extracts was observed in both dogs. Water balance was not detectably altered, and sodium balance did not change appreciably. A consistently low urinary specific gravity throughout the experiment affords further evidence that hydration was adequately maintained. In the dogs with diabetes insipidus, the serum potassium concentration was maintained at slightly higher levels than in the normal dogs during both the control and experimental periods.

Depletion experiments

Table II summarizes five balance studies in dogs depleted of sodium by peritoneal dialysis. In three of the four animals, detectable amounts of salt-retaining hormone appeared in the urine at some time during the study. The amounts excreted did not correlate well with the degree of hyponatremia, nor were they related to a further loss of body sodium or water, but appear to be more directly related to the level of the serum potassium. It is noteworthy that the largest amounts of hormone were generally excreted on the days when potassium intake was greatest (R, M, C).

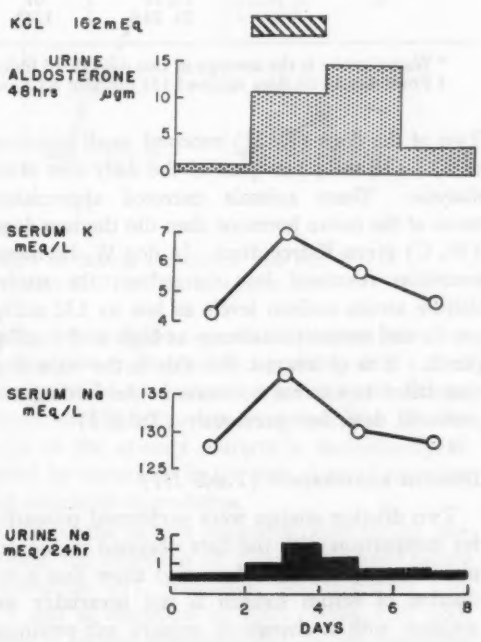

FIG. 3. EFFECT OF POTASSIUM FEEDING ON ALDOSTERONE EXCRETION AND SERUM POTASSIUM DURING SODIUM DEPLETION IN DOG R

Demonstration that sodium depletion with hyponatremia did not increase urinary aldosterone-like activity until dietary potassium was increased. No change in overall sodium balance was observed.

TABLE IV

Feeding experiments in dogs with diabetes insipidus

Dog	Experimental period	Consecutive days of study	Daily intake* Na (mEq.)	Daily intake* K (mEq.)	Daily intake* H_2O (ml.)	Observed serum Na (mEq./L.)	Observed serum K (mEq./L.)	Aldosterone activity 48 hr. (Est. μg.)	Average daily urinary excretion Na (mEq.)	Average daily urinary excretion K (mEq.)	Average daily urinary excretion H_2O (ml.)
T	Stock diet	1, 2			5,200	152	5.2	0	180	86	4,475
	Na and K free diet beginning on day 3	3, 4	0	0	2,050				15	18	1,780
		5, 6	↓	↓	1,850	152	5.0	0	17.5	14.5	1,800
		7, 8			1,150	156	4.9	0.24	7.0	12.0	1,070
		9, 10			1,450	152	5.8	0	6.0	12.0	1,450
	KCl added daily beginning on day 11	11, 12	0	67	2,150	153	4.8		6.0	70.0	1,520
		13, 14	↓	67	1,475	152	6.0	0.7	2.2	60.4	1,140
		15, 16		67	1,475	146	6.7	0.5	2.4	66.0	1,310
		23, 24		134†	2,550	150	7.6	2.0	3.8	122.0	2,300
E	Stock diet	1, 2			3,300	148	5.4	0	174.0	65.4	3,270
	Na and K free diet beginning on day 3	3, 4	0	0	1,325				15.9	24.3	1,375
		5, 6	↓	↓	1,225	151	5.4	0.01	6.5	7.6	865
		7, 8			1,185	150	5.6	0.7	4.9	6.5	1,322
		9, 10			1,800	149	5.4	0	2.7	3.9	1,595
	KCl added daily beginning on day 11	11, 12	0	67	2,500				3.8	46.8	2,275
		13, 14	↓	67	2,150	143	5.6	2.4	3.6	66.0	2,125
		15, 16		67	2,275	152	8.0	6.0	2.1	56.9	1,945
		23, 24		134†	3,315	149	7.7	3.0	7.0	120.4	3,180

* Water intake is the average of two days of ad libitum intake of distilled water.
† From day 16 on dogs received 134 mEq. of KCl and no Na in the daily ration.

Two of the dogs (R, M) received small amounts of KCl (18 mEq.) as part of the daily diet after dialysis. These animals excreted appreciably more of the active hormone than did the two dogs (W, C) given K-free diets. In dog W, hormone excretion remained low throughout the study, despite serum sodium levels as low as 132 mEq. per L. and serum potassiums as high as 5.5 mEq. per L. It is of interest that this is the same dog that failed to excrete hormone in the feeding experiment described previously (Table I).

Dilution experiments (Table III)

Two dilution studies were performed primarily for comparison with the data obtained in the depletion experiments. These also show that a reduction of serum sodium is not invariably associated with a change in urinary salt-retaining activity. In contrast to the dialysis depletion studies, the dilution procedure maintains the total body sodium more nearly at its normal level and hyponatremia is produced, at least in part, by excessive water retention. A parallel reduction in serum potassium also occurs. In one instance (N), when hypokalemia was corrected by feeding KCl, increased salt-retaining activity was noted in the urine. This occurred with hyperkalemia and an associated increase in total body water, as suggested by the fluid balance.

Figure 4 presents a graphic correlation between the levels of serum potassium and the excretion of aldosterone-like material in all of the animal studies. The response in a given animal appears to be qualitatively reproducible, as evidenced by the data obtained with dogs W and R.

Observations on a cardiac patient

The possible relationships between the levels of serum Na and K and the excretion of aldosterone have been explored in a man with heart failure and edema (Figure 5). The serum sodium concentration remained at about 120 mEq. per L. during the entire study. As in the case of the sodium-depleted dog, the serum potassium tended to be high, *i.e.*, 6 to 7 mEq. per L., when he was on a moderately high potassium intake. The serum potassium was reduced to 2.7 mEq. per L. over a 24-hour period by a single 2-ml. dose of

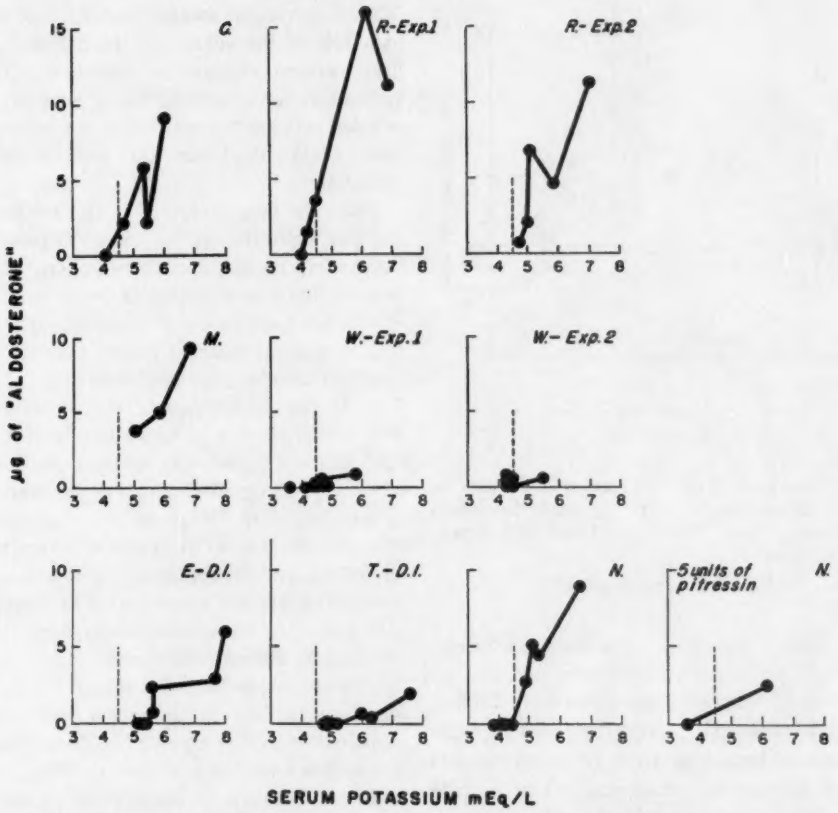

FIG. 4. CORRELATIONS BETWEEN THE CONCENTRATION OF SERUM POTASSIUM AND THE EXCRETION OF ALDOSTERONE-LIKE MATERIAL IN DOGS C, R, M, W, E, T, AND N

Mercuhydrin® in conjunction with the institution of a low potassium diet. As shown in Figure 4, the daily aldosterone excretion was initially high (300 μg.) and fell to a minimal value of 35 μg. per day after the serum K was reduced. This rate of excretion is still considerably above that observed in normal individuals on low sodium diets (9). Subsequently, the administration of K restored the serum K to higher levels and the excretion of aldosterone increased to a peak of 630 μg. per day. In this study, the estimates of aldosterone were confirmed by re-assay after chromatography and by U-V absorption and reduction with blue tetrazolium.

DISCUSSION

In this study, the amount of salt-retaining activity of urinary extracts has been considered to be an index of the rate of aldosterone secretion. It should be appreciated that probably only a small fraction of the total amount secreted by the adrenal cortex actually appears in the urine (8). The activity of the urinary extracts is undoubtedly affected by variations in the rate of renal excretion and metabolic degradation.

Among the factors suggested thus far as promoting the secretion of aldosterone are sodium deprivation (9), potassium administration (16, 17) and a reduction in body water (17, 18). There is general agreement that sodium restriction results in an increase in urinary aldosterone (8, 17) and, conversely, that the administration of sodium in sufficiently large quantities suppresses the excretion of aldosterone (8, 17, 19). Luetscher and Johnson (20) have reported an inverse relationship between urinary sodium and aldos-

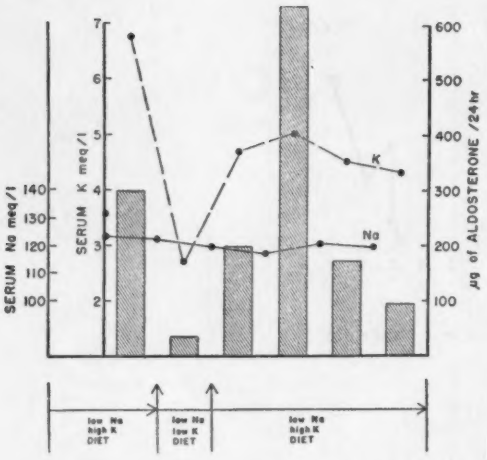

FIG. 5. METABOLIC BALANCE STUDY OF A PATIENT IN CONGESTIVE HEART FAILURE, WITH APPARENT RELATIONSHIP BETWEEN SERUM POTASSIUM LEVEL AND ALDOSTERONE EXCRETION.

Serum Na level did not change appreciably.

terone content. The effects of potassium administration have been reported by several groups (16, 17). It is of interest that Falbriard, Muller, Neher, and Mach (21), who administered potassium to normal human subjects, found an increase in urinary aldosterone which was not necessarily correlated with a reduced urinary sodium content. Further evidence that potassium may be important in aldosterone secretion has been obtained in the following experiments. Vogt (22) has reported an increase in the cold survival activity of the adrenal effluent during perfusion with solutions high in potassium which was not observed with perfusion of solutions low in sodium. In similar experiments utilizing the perfusion of calf adrenals, Rosenfeld, Rosemberg, Ungar, and Dorfman (23) demonstrated an actual increase in aldosterone and attributed this response to a lowering of the Na/K ratio in the perfusing fluid. Singer and Stack-Dunne found a reduction in adrenal venous aldosterone in rats depleted of potassium (24). Stoerk, Knowlton, and Loeb have found a correlation between the serum potassium and the size of the zona glomerulosa of rat adrenal cortex (25).

Bartter, Liddle, Duncan, and Delea (17) on the basis of studies involving water-loading or dehydration by various means, have stressed the dominant role of the volume of the extracellular fluid in governing aldosterone secretion. These investigators have been unable to establish any correlation between the electrolyte composition of the extracellular fluid and the rate of aldosterone elaboration.

From the data presented in this communication it appears that the administration of potassium has little effect on aldosterone excretion unless there is a simultaneous elevation of the serum potassium. It also has been shown that potassium administration in sodium depleted subjects causes a significant and sustained hyperkalemia (10, 11). Therefore, it may be anticipated that sodium deprivation, in the absence of potassium deprivation, may lead to some elevation of serum potassium. Consequently, changes in the rate of aldosterone secretion occurring in the course of sodium deprivation may not be entirely referable to alteration in sodium balance. In this connection, it is of interest that in the two subjects reported by Luetscher and Axelrod (9), the increases in urinary aldosterone induced by sodium withdrawal were accompanied by rises in the serum potassium of 0.7 to 0.9 mEq. per L., while the concentration of serum sodium remained relatively constant. More recently, this group has found with sodium deprivation that the extent of increase in hormone is directly related to the concurrent K intake (26).

In the present studies it is worthy of note that changes in aldosterone excretion did not appear to correlate well with any observable change in the level of serum sodium, sodium balance, or the state of hydration.

In edematous states, the administration of sodium (17, 19) or potassium deprivation, as reported here in one patient, may reduce urinary aldosterone but to values which remain well above normal. Furthermore, in experimental edema characterized by increased urinary aldosterone (27) a sustained large increase in plasma volume and in body water does not lead to sodium excretion as it does in normal subjects (15), nor is there evidence of a contracted volume in clinical edema. Thus, the increased urinary aldosterone of edematous states does not appear to be entirely accounted for by any of these known mechanisms.

SUMMARY

Metabolic balance studies have been carried out on dogs, some of which were depleted of sodium by peritoneal dialysis. Normal dogs did not exhibit any detectable salt-retaining activity in 48-hour urinary extracts when on diets containing normal amounts of sodium. Potassium administration in this situation did not induce appreciable hyperkalemia and was not associated with any detectable hormone activity of the urine.

Dietary restriction of sodium did not lead to appreciable hormone activity of urine extracts unless potassium was provided in the diet. With the feeding of potassium supplements the hormone activity increased.

Reduction in the serum sodium by peritoneal dialysis was associated with only slight to negligible hormone activity unless potassium was provided in the diet.

Two dogs with diabetes insipidus exhibited responses similar to the normal dogs.

The sodium-retaining activity of the urinary extracts in this study can be correlated with an induced increase in the serum potassium ion concentration and does not correlate well with reduction in the serum sodium ion concentration.

The data support the concept that an increase in the concentration of the serum potassium ion is a stimulus for the secretion of aldosterone.

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PHYSICAL BINDING OF INSULIN BY GAMMA GLOBULINS OF INSULIN-RESISTANT SUBJECTS [1, 2]

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The occasional occurrence of resistance to insulin among certain diabetic patients has been recognized for many years. However, the immunologic nature of this resistance, which may require the administration of over 1,000 units of insulin per day, has been demonstrated only by indirect means (2). Serum of insulin-resistant patients has been shown to protect mice from the hypoglycemic effect of insulin (3, 4), and to prevent the *in vitro* action of insulin on the rat diaphragm (5). The insulin-neutralizing property of such serum has been shown by electrophoretic (6, 7) and salt (8) fractionation to reside in the gamma globulin fraction. The substances in the plasma, presumably antibodies, related to the capacity of insulin to produce urticaria and allied reactions are apparently distinct from the insulin-neutralizing substances, and have been shown to reside in the beta globulin fraction (6).

Using I^{131}-labeled insulin, this study has shown that serum of insulin-resistant subjects, but not of normal individuals or non-resistant diabetic patients, complexes insulin and that this complex migrates at the leading edge of the gamma globulin fraction upon paper electrophoresis. By adding progressively larger amounts of insulin, it was possible, in most cases, to estimate the concentration of insulin needed to saturate such sera. Seventeen serum specimens from four insulin-resistant persons and one insulin-resistant rabbit were found to bind insulin in amounts from 0.05 μgm. to more than 20 μgm. per ml.

MATERIALS AND METHODS

Preparation of labeled insulin. Insulin labeled with I^{131} and containing 3 μc. of I^{131} per μgm. and about 0.6 atom of iodine per 24,000 molecular weight was prepared by a modification of the basic method of Banks, Seligman, and Fine (9). A mixture was made of 0.15 ml. of KI, containing 15 μgm. I, with 0.02 ml. of KIO_3 containing 3 μgm. of I in a 2-ml. glass stoppered tube. To this was added KI^{131} containing 14.8 μc. and about 0.12 μgm. of I followed by 0.1 ml. of 1 N HCl. After the addition of CCl_4 the contents were shaken periodically for 10 to 15 min., during which time about 80 per cent of the I was extracted as I_2 into the CCl_4 layer. The upper (aqueous) layer was removed by a dropper and 0.1 ml. of 0.12 N Na_2CO_3 containing 1 mg. crystalline bovine insulin [4] was added. The tube was shaken and allowed to stand for one hour.

Unreacted iodine was removed by treatment with an anion exchange resin. The insulin solution was diluted with 0.4 ml. of pH 7.4 buffer, containing 0.11 N NaCl and 0.04 M veronal, and placed on a 0.8 × 12 cm. column of Amberlite IRA 410, which had been saturated with HCl and adjusted to pH 7.4 prior to use. The insulin was washed through the column with the buffer, and finally made to 10.0 ml. with buffer. Yield was 20 per cent of the I^{131} used. Less than 4 per cent of the radioactivity of the insulin could be removed by prolonged dialysis. The I^{131}-labeled insulin was used within a week of its preparation to minimize the possibility of radiation damage to the insulin. At concentrations less than 10 μgm. per ml. the insulin was found to adsorb appreciably onto glass containers, so it was not diluted until just before use.

Testing procedure. After 0.02 ml. of labeled insulin solution, containing from 5 to 100 μgm. of insulin per ml. was added to 0.2 ml. of serum, 0.02 ml. of this mixture, or 0.2 μgm. of insulin, was applied in a narrow band across a 3-cm. strip of Whatman No. 3MM paper in the electrophoresis apparatus. A similar volume of the mix-

[1] Supported by AEC Contract At (30-1)-919 between the Atomic Energy Commission and the Massachusetts Memorial Hospitals.

[2] This work has appeared in abstract form (1), and was presented at the New England V.A. Clinical Research Society Meeting, Sturbridge, Massachusetts, October 7, 1955.

[3] Present address: The Mary Imogene Bassett Hospital, Cooperstown, New York.

[4] Armours 5 × recrystallized bovine insulin, graciously supplied by Dr. E. G. Ball of the Department of Biological Chemistry, Harvard Medical School.

ture was delivered into 1 ml. of water for subsequent determination of the exact amount of I^{131}-insulin present by gamma ray counting. The paper strips had been previously wet with the buffer (veronal, pH 8.6, ionic strength 0.05) and allowed to equilibrate for 20 to 40 minutes in the apparatus. The electrophoresis equipment was of a conventional type employing horizontally suspended strips, with a free length of 38 cm. between electrode baths. About 5 v. per cm. of free length were applied for 14 to 18 hours at room temperature. The strips were then dried in an oven at 95° C for 30 minutes.

RESULTS

In Figure 1 are shown typical results on different types of sera. Insulin labeled with radioiodine was detected by exposing the strips against X-ray film for 1 to 8 days. Proteins were subsequently detected by staining with 0.25 per cent bromphenol blue in 9/1 methanol/acetic acid, and rinsing in 30/3/1 water/acetic acid/phenol.

The findings agreed with those of Kallee (10, 11) in that insulin in these low concentrations, whether tested alone or mixed with normal serum, did not migrate upon electrophoresis, but remained adsorbed to the paper at the starting point (Figure 1, strips 1 and 2). Migration of free insulin could be obtained by adding a large excess of insulin, also shown by Kallee. In these cases the I^{131}-insulin migrated with a mobility about equal to that of serum albumin, but exchange between the labeled and non-labeled insulin and adsorption along the strips resulted in smearing and trailing of the labeled insulin. Hence, the more precise test for binding of insulin was to examine strips for movement of insulin which otherwise would have remained adsorbed at the starting point.

Strips 3, 4 and 5 of Figure 1 show binding of the I^{131}-insulin by the sera of three patients who were resistant to insulin. In strips 3 and 4 the insulin is completely bound so that none remains at the starting point. In strip 5 the serum has apparently been saturated, so that only a portion of the insulin moves. Insulin which is bound moves with the leading portion of the gamma globulins, suggesting that the insulin is complexed by gamma globulins into a soluble molecule with a mobility intermediate between the mobilities of free insulin and free gamma globulin.

In the detailed studies of Singer and Campbell (12, 13) on the physical chemical properties of soluble antigen-antibody complexes of ovalbumin and bovine serum albumin with their rabbit antibodies, it was shown that the electrophoretic mobilities of complexes were intermediate between

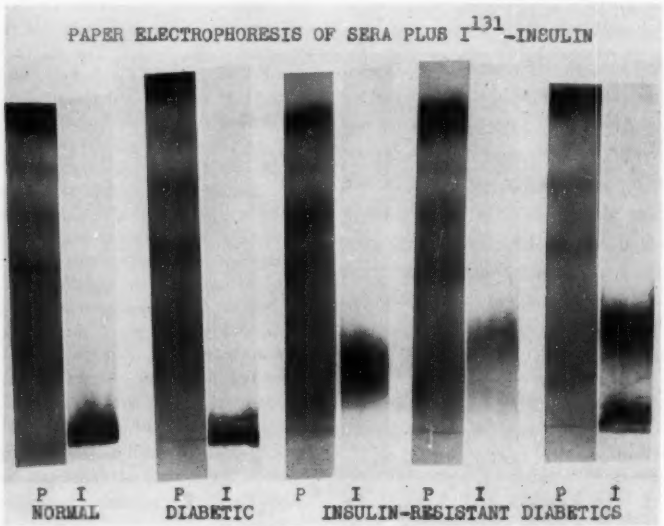

FIG. 1. ELECTROPHORESIS OF I^{131}-INSULIN MIXED WITH VARIOUS SERA

Strips labeled "P" are stained to show the protein fractions. Radioautographs of the same strips are labeled "I."

the mobilities of the free antigen and antibody, and could be predicted on the basis of the weight fraction of each component in the complex. From rough measurements of the relative mobilities on paper of free insulin (on insulin-treated papers), gamma globulin, and complexed I^{131}-insulin, it can be calculated on this basis that insulin comprises only 7 to 13 per cent of the weight of the complex. Assuming a molecular weight of 170,000 for the gamma globulin, this would mean that the complex contains one-half to one insulin molecule of molecular weight 24,000 per one gamma globulin molecule.

Quantitative measurements

For routine testing, radioautographs were not made, but the labeled insulin was located by passing the strip under a 1.9 mg. per sq. cm. end window Geiger counter, equipped with a lead cover containing a 0.5-cm. slit. Radioactivity was measured every 0.5 or 1 cm. of length along the paper strip, and the curve of radioactivity versus distance along the strip plotted. Movement of the labeled insulin could be detected by comparing the curve with the strip after staining to locate the protein fractions. When only part of the insulin migrated, the amount which migrated was estimated by determining the areas under the two curves with a planimeter.

In varying the insulin concentration to determine the saturation limit of the sera, the insulin concentration was not allowed to exceed 10 μgm. per ml. of serum. To avoid exceeding this level, the sera under investigation was diluted with varying amounts of serum from normal individuals. This also avoided the possibility of exceeding the saturation limit of the paper, when insulin would migrate in the free form.

Table I shows the results with various types of subjects. Eight normal persons and seven diabetic patients who were not resistant to insulin showed no migration of I^{131}-insulin with the gamma globulins, the level of detection being approximately 0.02 μgm. or 0.0007 unit of insulin per ml. serum. The sera of four insulin-resistant patients, and of a rabbit immunized against crystalline beef and pork insulin by one of the authors in a previous study (14), showed binding of insulin in ranges of 0.4 to 20 μgm. per ml. Some of the specimens had been stored frozen, with several thawings, for periods up to 15 years, but still gave satisfactory electrophoretic patterns.

Occasional modifications of the response were noted, such as a suggestion of a peak trailing the serum proteins (Figure 1, strip 4), or "tailing" or movement of the baseline. These modifications were seen occasionally with sera of resistant patients when large amounts of insulin were added, and were never seen with normal sera or with sera of diabetic patients who were not resistant to insulin, except in one specimen from a diabetic patient which showed a slightly increased tailing of the baseline. These effects may represent partial binding by serum with weak forces of the same order as the adsorption affinity of insulin for the paper.

With one patient it was possible to compare the amounts of insulin bound with the insulin requirement and the results of mouse protection tests on the same serum specimens (Table I). The periods of resistance to insulin as shown by the requirement for insulin and mouse test correspond with periods of high binding of insulin by the gamma globulins.

Species specificity

Table II shows evidence of species specificity of the insulin binding, when tested by adding nonlabeled human insulin in addition to the labeled bovine insulin to sera from insulin-resistant subjects. The amount of binding of I^{131}-bovine insulin which was observed was not reduced by the addition of human insulin to the degree expected if the two insulins were equally bound. This preferential binding of I^{131}-bovine insulin is in agreement with the finding (15, 16) that human insulin was effective in controlling the blood sugar in patient A. M. when large doses of bovine insulin were ineffective and in lowering the blood sugar in Rabbit No. 5 (14) when equivalent or larger doses of bovine insulin had no effect.

Recent reports have indicated (17, 18) that sera of insulin-treated diabetic patients, who showed no clinical resistance to insulin, bound labeled insulin either *in vivo* or *in vitro* so that it migrated with or just ahead of the gamma globulins. The present study has not demonstrated binding of insulin by gamma globulins in any of

TABLE I

I^{131}-insulin bound by serum proteins

Subject	Date	Clinical insulin resistance*	Insulin dose administered†		Insulin neut. by mouse test	Insulin bound by gamma glob. (*μgm./ml.*)
			Units/day	Type		
8 normals			0			0
7 diabetics			0–46	NPH		0§
	2/11/42	?0	0	Reg.	0	0.05
	3/2/42	+	800+	Reg.	+	10+
A. M.	8/18/42	?0	35	Reg.	?	0.05
	11/4/42	0	35	Reg.	0	0 (tailing)
	4/19/44	0	0–96‡	Reg.	0	0 (tailing)
	5/25/44	+	280+	Reg.	+	20
	4/13/44	+	ca. 300	Reg.		0.8+
C.	9/10/44	+	ca. 300	Reg.		0 (tailing)
	12/17/44	+	ca. 300	Reg.		1+
	9/7/44	+	ca. 500	Reg.		1.5
	9/21/44	+	ca. 500	Reg.		1.2
M.	4/15/48	+	ca. 500	Reg.		0.6+
	8/8/55	?	100	Reg.		0.14
O.	3/23/54	+	ca. 300	Reg.		0.2
	4/24/54	+	ca. 300	Reg.		0.4
Rabbit "No. 5"	9/26/47	+	(Immunized)	Cryst.		0.2
Rabbit "No. 5" (14)	3/23/48	+	(Immunized)	Cryst.		0.4

* Presence of clinical resistance indicated by "+", absence of resistance by "0". A "?" indicates that no direct test for resistance was done, but because of considerations discussed elsewhere (2), resistance was probably present or absent as indicated.

† The amount of insulin given does not necessarily reflect the insulin requirement, in that the patient may not have been completely controlled by the insulin dose indicated.

‡ Insulin therapy resumed two days previously, 31 to 96 units per day.

§ One specimen showed slight tailing of the adsorbed band.

the seven diabetics who were not resistant to insulin. No obvious reason could be found for this difference in results, but it might be related to 1) difference in sensitivity of detection of binding, 2) the use of bovine insulin in this study instead of the commercial mixture of beef and pork insulin or 3) lower antigenicity of the NPH-insulin received by all but one of the non-insulin-resistant diabetic patients reported here.

TABLE II

Preferential binding of I^{131}-bovine insulin in the presence of human insulin

Subject	I^{131}-bovine insulin		Human insulin added (*μgm./ml.*)
	Added (*μgm./ml.*)	Bound (*μgm./ml.*)	
O. 3/23/54	0.48	0.24	0
	0.44	0.23	0.44
A. M. 5/25/44	9	9	0
	60	22	0
	4	3.1	35
Rabbit No. 5	0.48	0.15	0
	0.54	0.14	0
	0.44	0.11	0.44

The technique described offers a sensitive method of testing for the presence of non-precipitating antibodies. It is somewhat similar to the technique of precipitation of labeled antigen with ammonium sulfate employed for this purpose by Farr (19), but differs from the paper electrophoresis technique used by others (20) for the detection of antibodies against DNP-beta-lactoglobulin, which depended upon the complexed antigen being rendered immobile by antibodies, apparently precipitin in nature. The above technique may be of value in testing for antibodies to protein hormones other than insulin.

SUMMARY

1. When I^{131}-labeled bovine insulin was added *in vitro* to serum from insulin-resistant persons the insulin was found to migrate with the leading edge

of the gamma globulin zone upon paper electrophoresis. Similar migration was not seen with serum from normal persons or diabetic patients who were not resistant to insulin. This suggests that the insulin resistance was due to the presence of antibodies which bound insulin into an inactive but soluble complex.

2. Insulin binding capacity amounted to 0.05 to more than 20 μgm. per ml. of serum. Evidence that human insulin was not bound in this manner is presented.

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PLASMA PROTEIN SYNTHESIS IN THE HUMAN FETUS AND PLACENTA [1]

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Electrophoretic studies of human fetal plasma have demonstrated a protein pattern in which all major fractions are present (1). The site of origin of these plasma proteins is not known. Presumably they may be synthesized by the mother and merely circulate in the fetus as the result of placental transfer, or they may be synthesized by the placenta or the fetus.

The gamma globulin level is high at birth and falls during the first weeks of life (2). This led to the assumption that the gamma globulin found at birth is derived from the mother by transfer through the placenta, and that the newborn infant does not synthesize it. Because gamma globulin levels (2) and antibody levels (3) are often higher in cord blood than in maternal blood there has been speculation that the placenta may be the site of synthesis. The problem of plasma protein synthesis in the placenta has been approached by comparing levels of the various fractions in blood from the umbilical artery and vein and from the mother (4). It was concluded that the placenta synthesizes albumin.

The present experiments were designed to investigate the synthesis of plasma protein by placenta and fetal liver. Term placenta was obtained at normal deliveries. Liver and placental tissues were also taken from three fetuses of 3 to 4 months gestation (Table I). For purposes of comparison fetal heart was similarly investigated. The fetuses were delivered surgically for psychiatric and social indications and were presumably normal. Tissue slices were incubated with glycine-2-C_{14}, and the incorporation of radioactivity into plasma proteins was studied. The proteins were isolated and identified electrophoretically and by immunological techniques.

PROCEDURE

Incubation

Tissues were procured immediately after delivery and dropped into chilled isotonic saline. Slices about 0.5 mm. thick were prepared with a Stadie-Riggs slicer. One gram of tissue was added to 2.5 ml. of Krebs-Ringer-bicarbonate buffer (5) in a 25-ml. flask to which had been added 5 micrograms of Aureomycin and about one million counts per minute of glycine-2-C_{14} (Tracerlab). Radioactivity was determined as described below. The slices were incubated with constant shaking for 18 hours in a water bath at 37° C.

Electrophoresis

At the end of incubation, the tissue slices were centrifuged down and discarded. Incubation media from each type of tissue were pooled and dialyzed against three changes of isotonic saline during 24 hours at 5° C. The bag contents were lyophilized and redissolved in 1 ml. isotonic saline to reduce to volumes suitable for electrophoresis. The samples were dialyzed for three hours against veronal buffer, pH 8.6, and then separated by starch electrophoresis and the protein pattern developed as described by Kunkel and Slater (6). One ml. of human plasma was run in parallel on the same block to serve as marker for the plasma protein fractions.

To determine the radioactivity curve, the protein was precipitated from an aliquot of each eluted fraction by the addition of equal volumes of 10 per cent trichloracetic acid (TCA). The precipitate was washed with 5 per cent TCA and extracted with ether to remove the TCA. The precipitate was redissolved in 0.05 N NaOH, transferred to a planchette, and the radioactivity determined.

Immunological identification of radioactive proteins

Liver. Carrier precipitates were made in eluates from the starch electrophoresis by adding various plasma fractions and their specific antisera prepared in rabbits. Fractions 5 to 12 (Figure 1), representing the alpha and beta globulins, were pooled, divided into five aliquots, and

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[2] John and Mary R. Markle Scholar in Medical Science. A large part of the work was done while Dr. Dancis was a Lowell Palmer Senior Research Fellow.

TABLE I

Data on fetuses

Fetus	Weight (*grams*)	Gestation* in days (history)	Gestation† in days (from weight)
I	150	130	120
II	140	195	120
III	240	130	130

* Calculated from menstrual history.
† Estimated from weight according to graph by Widdas (18).

a specific precipitate of about 2 mg. was made in each aliquot. A preliminary titration was done in a small sample of each aliquot, so that appropriate amounts of antigen and antibody were added to produce the precipitate at or near the equivalence point. Precipitates were prepared with the following fractions: human albumin, guinea pig albumin, human beta globulin, beta lipoprotein, and metal-combining globulin. Similar precipitates were prepared in the albumin fraction (17 and 18, Figure 1). All of the specific precipitates were made in aliquots of the eluates containing approximately the same amount of radioactivity. This permits comparison of the amount of radioactivity incorporated in each specific precipitate.

The fibrinogen was located in fractions 12 to 14 by adding thrombin to the concurrently separated human plasma. A precipitate was then formed in fractions 12 to 14 of the incubating medium by adding carrier fibrinogen and the specific antiserum. A similar control precipitate was made in fractions 16 to 18.

The precipitates were washed with saline, dissolved in concentrated ammonium hydroxide, and transferred to planchettes for determination of radioactivity.

Heart. Because of insufficient material, only electrophoretic separation could be done in the first experiment. In the second experiment, a preliminary separation was performed by dialysis against 1.75 M ammonium sulfate (7). The precipitate, containing all the gamma globulin and a fraction of the other globulins, but little albumin, was redissolved in saline. Immunological precipitates were made in the "albumin" fraction (supernatant) and the "globulin" fraction.

Placenta. After electrophoretic separation, it was demonstrated that the radioactive proteins were distributed in the alpha and beta globulin fractions. These fractions were separated (Figure 3, fractions 5 to 9 and 10 to 14, respectively), and specific precipitates were made in each fraction.

In earlier experiments with term placentas the technique described by Keston and Katchen was used (8). After incubation the tissues were homogenized and the mixture centrifuged. The supernatant was dialyzed against saline and specific precipitates were made in aliquots of the supernatant without preliminary electrophoresis. To provide an estimate of non-specific adsorption by the protein precipitates, specific precipitates were made

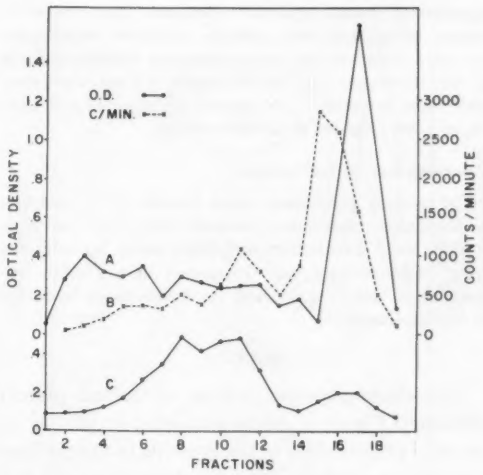

FIG. 1. SYNTHESIS OF PROTEINS BY FETAL LIVER
A: Electrophoretic pattern of human plasma proteins.
B: Total radioactivity of proteins in the incubating medium. Note similarity to A.
C: Pattern of proteins in incubation medium.

with physically similar but heterologous proteins (ovalbumin, guinea pig albumin, and bovine gamma globulin).

Preparation of specific antisera

Antisera were prepared in rabbits by the injection of plasma protein fractions with Freund's adjuvant (9). Most of the antisera were prepared by injecting interdigitally 3 to 5 mg. of antigen emulsified in 0.5 ml. Falba, 1 ml. Bayol F and 1 mg. of killed tubercle bacilli. Some of the antisera prepared early in the study required 50 mg. of antigen in 4 ml. of adjuvant, and the injections were intramuscular.

The human gamma globulin and fibrinogen were obtained from the Department of Biophysical Chemistry, Harvard University. The gamma globulin was electrophoretically pure. The antiserum to fibrinogen was purified by absorption with human serum.

The antisera against beta lipoprotein and metal-combining globulin were generously supplied by Dr. Leonhard Korngold of the Sloan-Kettering Institute for Cancer Research. The preparation of the former and the tests for specificity have been described (10). The latter was prepared by injection of the crystalline antigen and absorption of the antiserum with human gamma globulin and albumin.

The human albumin was supplied by Dr. T. W. Green of Cutter Laboratories, Berkeley, California. It was stated to be 98 per cent electrophoretically pure with the major contaminant alpha globulins. Bovine gamma globulin was obtained from Armour and ovalbumin from Dr. Bernard Katchen.

The beta globulins were prepared by starch electro-

phoresis of human plasma. Antisera were prepared against alpha and beta globulin fractions individually but were found to be immunologically indistinguishable by the techniques used in this study. They were then used interchangeably. The guinea pig albumin and antiserum were prepared in similar fashion.

Determination of radioactivity

The protein precipitates were transferred in solution to aluminum planchettes covered with filter paper to produce even distribution, and dried under an infra-red lamp. Radioactivity was determined in a D-46A Nuclear Flow Gas Counter, and the counts were corrected to infinite thinness.

RESULTS

The electrophoretic pattern of normal plasma proteins (Figure 1 A) is paralleled strikingly by the total radioactivity of the proteins in the medium used for the incubation of *liver* slices (Figure 1 B), except that there is no incorporation of radioactivity into proteins with gamma globulin mobility. The electrophoretic pattern of the proteins in the incubation medium (Figure 1 C), probably representing primarily liver proteins, is quite dissimilar.

Carrier precipitates were made by adding various plasma proteins and their specific antisera to fractions of the electrophoretically separated proteins, as previously described. The results are presented in Table II. It is evident that a significant amount of radioactivity is carried down by a specific precipitate prepared in the appropriate fraction (*e.g.*, the human albumin precipitate in the albumin fraction), and that it is considerably less in the inappropriate portions of the curve (*e.g.*, the albumin precipitate in the globulin fraction). Precipitates prepared with guinea pig albumin and its specific antiserum, to give an index of non-specific adsorption, also contain much less radioactivity. The gamma globulin precipitates seemed to give non-specific results. This suspicion was verified by demonstrating extensive cross-reaction between human albumin and our gamma globulin antiserum. The gamma globulin precipitates also incorporated as much radioactivity as the beta globulin precipitates even though the radioactive proteins had an electrophoretic mobility corresponding to the latter.

TABLE II

Incorporation of radioactivity into proteins by fetal liver (counts per minute) *

Precipitates	Experiment II		Experiment III	
	Globulin fraction	Albumin fraction	Globulin fraction	Albumin fraction
Total protein†	1594	1854	1210	915
Albumin (human)	95	1130	51	980
Beta globulin	670	44	598	13
Metal-combining	171	10	148	2
Lipoprotein	239	32	279	18
Gamma globulin	688	566	560	169
Albumin (guinea pig)	58	26	27	50
	Fibrinogen fraction	Fibrinogen control	Fibrinogen fraction	Fibrinogen control
Total protein†	574	890	474	572
Fibrinogen	186	78	201	105

* Carrier precipitates were formed by adding the protein and its specific antiserum to appropriate electrophoretic fractions (*e.g.*, albumin precipitate in albumin fraction) and, as a control, in inappropriate fractions (*e.g.*, albumin in globulin fraction). Guinea pig albumin precipitates are further controls.

† TCA precipitable radioactivity.

The results of the experiments with *fetal heart* are presented in Figure 2 and Table III. The electrophoretic pattern of the radioactive proteins in the incubation medium (Figure 2 B) resembles more closely that of the presumed tissue proteins (C) than the plasma proteins (A). The amount of radioactivity incorporated is much less than in the liver experiment (note the difference in scale in Figures 1 and 2).

Unfortunately there was insufficient material after electrophoretic separation to permit immunological identification. Accordingly, in a second experiment, preliminary separation was done with ammonium sulfate. There is a significant amount of radioactivity in the beta globulin and albumin precipitates made in the supernatant (Table III). However, the results with guinea pig albumin in-

TABLE III

Incorporation of radioactivity into specific precipitates by fetal heart (counts per minute) *

	Globulin fraction	Albumin fraction
Total protein	107	124
Albumin (human)	0	56
Beta globulin	10	40
Gamma globulin	8	
Albumin (guinea pig)		38

* Incubation medium proteins were separated by ammonium sulfate precipitation into two fractions and specific precipitates made in each fraction.

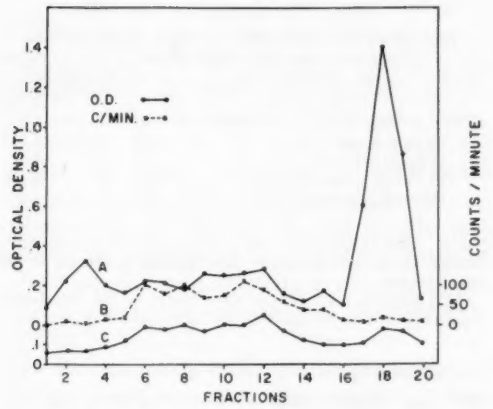

FIG. 2. SYNTHESIS OF PROTEINS BY FETAL HEART
A: Electrophoretic pattern of human plasma proteins.
B: Total radioactivity of proteins in the incubating medium.
C: Pattern of proteins in incubation medium. Note similarity of B and C in this figure.

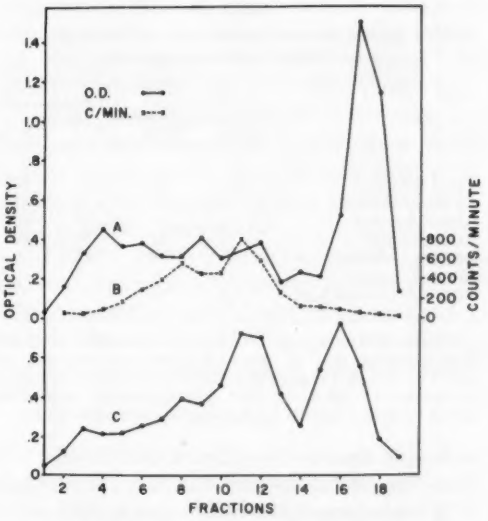

FIG. 3. SYNTHESIS OF PROTEINS BY TERM PLACENTA
A: Electrophoretic pattern of human plasma.
B: Total radioactivity of proteins in the incubating medium.
C: Pattern of proteins in incubation medium.

dicate that this is probably because of non-specific adsorption.

The results of an experiment with term *placenta* are given in Figure 3. The total radioactivity of the proteins in the incubating medium (B) parallels the protein pattern (C) more closely than that of the plasma proteins (A). There is no radioactivity in proteins with albumin or gamma globulin mobilities. It is presumed that the albumin peak in C is derived from blood in the tissue slices since the placenta apparently does not synthesize proteins with this mobility (B).

The results of similar experiments with placentas from 3 to 4-month pregnancies are not reproduced here because there are no important differences. The radioactive proteins moved primarily in the beta and alpha globulin zones. There were no radioactive proteins with albumin mobility, but a small amount overlapped the faster moving gamma globulins. As with term placenta the pattern resembled more closely that of the proteins of the incubating medium than that of plasma proteins.

Carrier precipitates were made by adding plasma proteins and the specific antisera to the alpha and beta globulin fractions (Figure 1, fractions 5 to 9 and 10 to 14, respectively). In the early placenta, the amount of radioactivity in the precipitates was about the same as that obtained with guinea pig albumin, and probably represented non-specific absorption. However, in term placentas there was significantly greater radioactivity in the precipitates prepared with plasma globulins (Table IV).

In earlier experiments using immunological isolation of proteins without preliminary electrophoretic separation (Table V), there is more radioactivity in the gamma globulin precipitate than in the control precipitates. In the type of experiment presented in Table V A, the slices were homogenized in the incubating medium and the immunological precipitates made in the supernatant. In Table V B, the precipitates were made in the incubating medium without preliminary homogenization of the tissue. The reduction in non-specific adsorption is evident.

DISCUSSION

This investigation was originally undertaken to determine if the placenta could synthesize plasma proteins. The albumin and gamma globulin fractions were first investigated because reports from other workers had suggested that the placenta may synthesize these fractions. The

TABLE IV

Incorporation of radioactivity into proteins by placenta (counts per minute)

Protein	Early placenta*		Late placenta*	
	Beta globulins	Alpha globulins	Beta globulins	Alpha globulins
Total protein	213	260	368	443
Albumin (human)	7	7	0	25
Alpha globulin			30	105
Beta globulin	4	21	36	125
Gamma globulin	19	19	43	124
Albumin (egg)			8	2
Albumin (guinea pig)	18	14		

* Early placenta was from a 3 to 4 months gestation; late placenta was at term. Carrier precipitates were made by adding the plasma protein fraction and its specific antiserum to aliquots after electrophoretic separation. Total protein refers to TCA precipitable radioactivity.

technique described by Keston and Katchen (8) (see Procedure) seemed suitable, and in Table V A are presented the results of a typical experiment with term placenta. The amount of radioactivity incorporated into the gamma globulin precipitate appeared significant; however, the amount of radioactivity in the control precipitates was also appreciable. In a second series of experiments (Table V B), the specific precipitates were made in the incubating medium without preliminary homogenization of the tissue, since it seemed reasonable that the plasma proteins would diffuse out of the cell. This reduced the amount of radioactivity brought down in the control precipitates, verifying the negative results with albumin and making more significant the results with gamma globulin. Cross-reaction between bovine and human gamma globulin (11) probably accounted for the intermediate amount of radioactivity in the former precipitates. These results have been previously presented (12).

The technique of electrophoretic separation followed by immunological precipitation was resorted to in an attempt to better identify the proteins. The data obtained with fetal liver and heart will be discussed first, since they add to the interpretation of the results with placenta.

The experiments with liver give clear cut evidence that the fetus is already capable of synthesizing plasma proteins by 3 to 4 months gestation. The electrophoretic separations indicate that the liver is capable of synthesizing all of the electrophoretically identifiable proteins except gamma globulin. These results conform with those of

TABLE V

Incorporation of radioactivity into specific precipitates by term placenta (counts per minute)

	A*	B†
Egg albumin	75	6
Human albumin	77	4
Bovine gamma globulin	115	30
Human gamma globulin	355	72

* A: The tissue slices were homogenized in the incubating medium and carrier precipitates prepared in the supernatant.

† B: Carrier precipitates were made in the incubating medium without preliminary homogenization of tissues.

perfusion experiments with adult rat liver (13) and slice experiments with adult guinea pig liver (14). The immunological studies confirmed the identity of the proteins and gave further information about sub-fractions of the beta globulins.

The gamma globulin precipitates gave puzzling results. The radioactivity carried down in the albumin fraction was probably the result of cross-reaction. We later demonstrated extensive cross-reaction between our gamma globulin antiserum and albumin. However, the gamma globulin precipitates were just as effective as the β globulin precipitates in bringing down radioactivity among proteins with beta and alpha globulin mobility. This was also true in experiments with term placenta, as will be discussed below. It is well known that the plasma globulins are closely related immunologically (15). The question may be raised as to whether immunological differentiation is even less distinct at this early stage of development.

The results with fetal heart are clearly different from those obtained with liver, and conform with what might be expected from an organ that synthesizes proteins for its own use. The radioactive proteins that have diffused into the medium are small in amount and the electrophoretic pattern resembles that of the non-radioactive proteins in the medium, probably representing tissue proteins.

Because of the small amount of tissue available from fetal heart, and the low level of incorporation of radioactivity into proteins, there was insufficient material after electrophoretic separation to permit the preparation of immunological precipitates in the various fractions. In the second experiment, ammonium sulfate precipitation was employed to separate roughly the proteins. The albumin and beta globulin precipitates made in the supernatant,

after ammonium sulfate precipitation, had radioactivity. Since this fraction might be expected to contain the albumin and a large proportion of the beta globulins, and since there was also radioactivity in these fractions as determined electrophoretically, this finding might have been considered significant. However, a heterologous precipitate indicated that the radioactivity was incorporated by non-specific adsorption. The proportion of radioactivity brought down with the guinea pig albumin precipitate in this experiment may be contrasted with that in the fetal liver (Tables II and III).

In the experiments with term placenta, electrophoretic identification of the radioactive proteins demonstrated that the placenta did not synthesize proteins with either albumin or gamma globulin mobility (Figure 3 B). The proteins moved into the alpha and beta globulin area and appeared to be related immunologically to all three globulin fractions in that each carrier precipitate brought down approximately the same amount of radioactivity (Table IV).

Particular pains were taken with the gamma globulin precipitates because of the paradoxical results with electrophoretic separation. In a series of experiments, different antigens and different antisera were used. In one experiment the gamma globulin antigen was prepared by precipitating diphtheria toxoid with immune human serum, the precipitate being largely composed of the specific anti-diphtheria gamma globulin. The results in all experiments were essentially the same.

It would be of interest to know if the globulins synthesized by the placenta have functional similarities to plasma globulins. Good and Zak have presented an interesting report (16) of a woman with agammaglobulinemia who was immunized repeatedly during pregnancy. Antibodies were detected in the mother in the last months of pregnancy and circumstantial evidence indicated that the placenta might be the source of the antibodies. Since some antibodies have electrophoretic mobilities similar to those of the radioactive proteins in our experiments with placenta (17), we made attempts to demonstrate antibody synthesis using the Keston and Katchen technique with placentas from mothers immunized against tetanus and diphtheria. The attempts were unsuccessful. This does not exclude the possibility that the placenta synthesizes antibodies in amounts too small to be detected by this technique, or that it may synthesize antibodies under abnormal conditions (for example, agammaglobulinemia in the mother).

It is not likely that under normal circumstances the placenta contributes significantly to the plasma proteins of the fetus. The placenta from pregnancies of 3 to 4 months duration does not synthesize any plasma proteins detectable by this technique (Table IV). By this stage of pregnancy, the fetal liver is already actively synthesizing all plasma protein fractions except the gamma globulin. The gamma globulins as identified electrophoretically are probably supplied by the mother.

SUMMARY

1. Liver slices from human fetuses of 3 to 4 months gestation were incubated with glycine-2-C_{14}. The proteins were separated electrophoretically and immunologically, and the incorporation of radioactivity was determined. It was concluded that the liver at this stage of development is already capable of synthesizing plasma proteins, exclusive of gamma globulin.

2. Similar studies were conducted using human fetal heart for purposes of comparison. The differences were striking and conform with the picture of an organ synthesizing proteins for its own use.

3. Experiments with human placenta from pregnancies of 3 to 4 months duration have demonstrated that the placenta will incorporate glycine-2-C_{14} into proteins with alpha and beta globulin mobilities. These proteins are immunologically unrelated to plasma proteins.

4. Similar experiments with term placentas revealed incorporation of radioactivity into proteins with similar electrophoretic mobility. However, these proteins are related immunologically to plasma globulins.

5. At neither stage of pregnancy does the placenta synthesize proteins that are electrophoretically identifiable as albumin or gamma globulin.

6. It is concluded that under normal circumstances the placenta does not contribute significantly to the plasma proteins of the fetus. Beginning early in gestation, the fetal liver is capable of synthesizing all plasma proteins except gamma globulin. The gamma globulin is probably derived from the mother.

ACKNOWLEDGMENT

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ANTIBODY PROTEIN SYNTHESIS BY LYMPH NODES HOMO-TRANSPLANTED TO A HYPOGAMMAGLOBULINEMIC ADULT

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After showing that normal skin homografted upon a congenitally agammaglobulinemic child survived for a prolonged period (1), Good and Varco further demonstrated that within certain limits hypogammaglobulinemic patients might be artificially endowed with a system of active immunity through homotransplantation of a reticuloendothelial tissue (2, 3).

The present study was designed both to explore the therapeutic possibilities of lymph node homotransplantation and to take advantage of the unique antibody-synthesizing defect in hypogammaglobulinemia to quantitate the immune response of human lymphoid tissue.

This paper presents the results of measurements of antibody protein synthesis, during primary and secondary immune responses, by normal lymph nodes homotransplanted to a hypogammaglobulinemic adult, together with qualitative observations of the interplay of recipient and transplant during and after the 150- to 160-day period of survival of the lymph nodes.

MATERIALS AND METHODS

Challenge antigen. Because it is potent, reliable, and rapidly productive of antibodies susceptible to accurate measurement, typhoid vaccine was selected as the antigen with which to test the reactivity of the transplant. The preparation employed[1] to challenge both recipient and donor contained 1,000 million heat-killed organisms of the Panama 58 strain per ml., and was of the type ordinarily stimulating production of H and O (but not V_1) antibodies.

Titration of typhoid H- and O-agglutinins. Agglutinins were measured on coded specimens by one observer at one time, and expressed as the mean of four determinations on recipient sera and two on donor sera, by the slide agglutination method devised by Welch and Stuart (4) and improved by Diamond (5). Single pooled batches of commercial (Lederle) Salmonella Group D somatic antigen and of typhoid H antigen were employed. The test was further refined as follows: the titration was begun at 1:10, employing 0.16 ml. of serum, and all subsequent successively smaller serum aliquots were made up to 0.16 ml. with saline prior to the addition of 0.03 ml. of antigen; moreover, dilutions of 1:15, 1:30, 1:60, etc., employing appropriate serum aliquots, were interpolated between the conventional 1:10, 1:20, 1:40, etc. dilutions. Under these more rigorous conditions, end-points (50 per cent agglutination) were sharp and repeated titrations reasonably reproducible.[2] The readings obtained were deemed internally consistent, though not necessarily quantitatively identical with values ordinarily obtained with different batches of tube or slide antigens.

No satisfactory method of accurate titration of low ($< 1:10$) titers of H-agglutinins was found. Titers of O-agglutinins as low as 1:2.5, however, could be measured satisfactorily by prior 2- or 4-fold concentration of the serum beta$_2$-globulins through the use of zinc-proteinate reactions based on Cohn plasma fractionation Method 12 (6) as follows:

A portion of cold, buffered zinc diglycinate-zinc acetate solution, containing 500 mM Zn^{++} per L., was added to the serum at 0 to 2° C to a final Zn^{++} concentration of 20 mM per L. The mixture was allowed to equilibrate 30 minutes and was centrifuged at 0 to 2° C for 15 minutes at 1,400 *g*. The supernatant was discarded. The precipitate was made up to one-fourth or one-half the original serum volume with 0.3 M sodium citrate at pH 7.2. Recovery of both H- and O-agglutinins appeared to be complete, but the concentrate was suitable for slide agglutination titration of only O-antibody. The presence of citrate ion apparently inhibited the reaction of H-antigen and antibody; the citrate effect could be reversed by dialysis, but only at the expense of reduced concentration.

Titration of tissue antibody. Tissue extracts suitable for titration of H- and O-agglutinins were prepared as fat-free saline suspensions by the method of Mountain (7).

Immunochemical estimation of typhoid O-antibody. To define a ratio between units of serum typhoid O-agglutinin activity (reciprocal of titer) and micrograms of typhoid O-beta$_2$-globulin nitrogen per ml., the method of analysis of specific precipitates (8, 9) was adapted as follows:

The antigen used consisted of a saline suspension of the colonies scraped from a 20-hour agar slant culture

[1] Typhoid Vaccine, Eli Lilly and Co., Lot No. 7289-62783.

[2] See Table III.

of *Salmonella typhi*, H901W strain. The suspension was heated two and one-half hours at 100° C to destroy traces of flagellar antigen, was centrifuged, and was twice washed with saline. Saline was then added to yield a suspension containing approximately 60 μg. N per ml.

Preliminary experiments under conditions of antibody excess and antigen excess indicated a wide equivalence zone, as reported by Landy, Johnson, Webster, and Sagin (10), with prozone and postzone phenomena occurring only under extreme conditions; in addition, the very low antibody N: antigen N ratio in equivalence-point precipitates noted by Gurevitch and Ephrati (11) was confirmed.

Sera taken from donor and recipient were used in 0.2- to 5.0-ml. amounts. Sera were inactivated 30 minutes at 37° C and prior to antigen addition were centrifuged free of traces of particulate debris. Mixtures of serum, saline, and 1.0 ml. antigen were incubated 1 hour, with occasional agitation, in a 37° C bath, and then for 18 hours at 4° C.

The mixtures were spun 30 minutes at 3° C and 1,400 *g*. The supernatants were decanted and checked for residual agglutinating activity, and the precipitates were washed twice with cold saline and transferred quantitatively to Kjeldahl digestion flasks.

Nitrogen was determined by a micro-Kjeldahl procedure employing powdered selenium as a catalyst. The color reaction with Nessler's reagent was developed in the cold and read immediately at 500 mμ in a spectrophotometer.

Immunochemical estimation of typhoid H-antibody. The method described above was used, with the following differences:

Antigen was prepared by adding an equal volume of 0.6 per cent formalinized saline to a 20-hour trypticase-soy broth culture of the H901W strain of *S. typhi*, the motility of which had been increased to a maximum by repeated passage in semi-solid agar. The organisms were centrifuged and washed free of broth protein, and saline was added to yield a suspension containing approximately 50 μg. N per ml. The sera measured were those noted above, from which O-antibody had been completely absorbed.

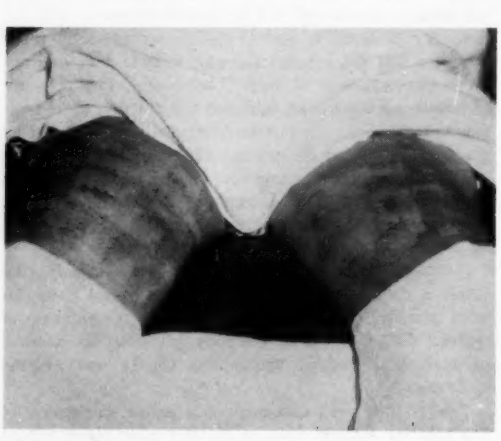

FIG. 1. SITES OF TRANSPLANTATION OF LYMPH NODE SLICES IN MEDIAL THIGHS

Tuberculin testing. Tuberculin tests of the recipient were performed with 0.1-ml. intradermal doses of fresh solutions of a single lot of Sharp and Dohme Second-Strength Tablets Tuberculin, Purified Protein Derivative (0.005 mg. per 0.1 ml.), and 0.1 ml. doses of fresh saline dilutions of that lot equivalent in potency to P.P.D. Intermediate (0.0001 mg. per 0.1 ml.) and P.P.D. No. 1 (0.00002 mg. per 0.1 ml.). Induration was measured by the method of Lovell, Goodman, Hudson, Armitage, and Pickering (12) at 24, 48, and 72 hours, and units of tuberculin reactivity roughly quantitated as millimeters mean maximum induration per log micro-microgram P.P.D., a unit based on the linear relationship between that measurement of the response and the logarithm of the dose, as described in man (12) and in animals (13).[3] Two dilutions were used for each skin test, and on each occasion the two responses agreed within 5 to 10 per cent.

Histological methods. Tissues for histological examination were fixed in 70–30 absolute alcohol-formalin solution and serially sectioned. Alternate sections were stained with hematoxylin-eosin and methyl-green-pyronine, and selected sections were stained with iron-hematoxylin, eosin-methylene blue, and Giemsa stain. Several sections were examined by polaroid and phase microscopy. Attempts to count plasma cells, reticulum cells, and lymphocytes were abandoned, since no one stain differentiated the cell types sufficiently well to insure accuracy on successive counts.

Leukocyte suspensions. Fresh suspensions of viable leukocytes suitable for skin-testing and for agglutinin studies were prepared from whole blood by the dextran-dextrose-sequestrene technique of Brecher, Wilbur, and Cronkite (16). For skin-testing, the .02 ml. of packed leukocytes derived from 10 ml. blood were resuspended in 0.1 ml. normal saline and injected intradermally. The same volume of leukocytes, washed and resuspended in 2.0 ml. saline, served as an antigen for slide agglutinations.

Statistical methods. In the construction of the curve which best fits the observed antibody titers, standard methods of graphic analysis of multiple linear regression curves were employed to resolve the changing slopes.

THE RECIPIENT, THE DONOR, AND THE CLINICAL CONDITIONS

Hypogammaglobulinemic recipient. The recipient of the transplant was a 64-year-old white woman with ac-

[3] Under this schema, maximum responses of 10 × 10 mm. induration to 0.1-ml. doses of P.P.D. No. 2, Intermediate, and No. 1 are equivalent, respectively, to 2.7 5.0, and 7.7 units of tuberculin reactivity; and the 1+, 2+, 3+, 4+ criteria employed by Lawrence in passive transfer experiments (14, 15) are equivalent, respectively, to 1.4–2.7, 2.7–5.4, 5.4–8.1, and > 8.1 units.

quired hypogammaglobulinemia,[4] whose case history has been reported elsewhere (17). In addition to her basic disease, the following secondary complications and unrelated conditions were present: a stable mediastinal mass (presumably a hyperplastic, now fibrotic, thymus); chronic pyelonephritis (with normal blood urea nitrogen and only mild impairment of renal function); bronchiectasis; hypersplenism (with a moderate hemolytic anemia and a cyclic neutropenia); and arteriosclerotic heart disease, with mild ankle edema despite therapeutic doses of digitoxin.

Throughout the 137-day period preceding the transplantation and the 231-day period following it, the recipient received sulfadiazine sufficient to maintain a near-constant serum sulfonamide level of 5 to 8 mg. per 100 ml. and human gamma globulin,[5] 5.0 gm. intramuscularly every 14 days, a dose which maintained a constant serum gamma-globulin level of 0.30 ± 0.02 gm. per 100 ml. (17) and a constant exogenous typhoid H-agglutinin titer of 1:10. She had previously proved totally unresponsive to primary and repeated booster doses of typhoid-paratyphoid vaccine and to several 0.1-ml. intradermal doses of P.P.D. No. 2.

There began to develop, 30 to 40 days prior to transplantation, a severe neutropenia which, instead of remitting as in the past, persisted until the 257th day after transplantation.

Choice of donor. The following major and minor criteria were formulated for the selection of a donor for safe and successful transplantation:

Major: 1) No evidence of active tuberculosis, of other acute or chronic transmissible infection, or of neoplastic disease; 2) Neither evidence of past or of present hepatitis nor history of transfusions within the past 6 months; 3) Close genetic relationship to the recipient; 4) Requirement of abdominal surgery for other, benign reasons, to which lymph node excision would be incidental.

Minor: 1) Identical blood type (B, Rh positive); 2) Positive tuberculin reaction (or other delayed-type cutaneous hypersensitivity); 3) Age between 20 and 50 years; 4) Absence of typhoid agglutinins and no history of typhoid infection or immunization.

The donor selected was the patient's 62-year-old sister, who had always been in good health and who required an elective hysterectomy and perineal repair for a third-degree uterine prolapse and cystocoele. Studies revealed Type A, Rh positive blood, normal liver function, a positive reaction to P.P.D. No. 1, and X-ray evidence of healed fibrotic disease at the apex of the right lung. There was no history of typhoid fever or of typhoid immunization, and typhoid H- and O-agglutinins were absent in 1:10 and 1:2.5 dilution, respectively. Titers of somatic agglutinins against other Salmonellae were: A: $< 1:5$; B: $< 1:5$; C (C_1, C_2): 1:40; and E (E_1, E_2, E_3): $< 1:5$.

Except in age and blood type, the donor therefore satisfied all the major and minor criteria.

Homotransplantation procedure. On June 22, 1955, a left supraclavicular lymph node was excised from the recipient. One-half was fixed promptly in alcohol-formalin and the other frozen for later antibody studies.

Simultaneously, in an adjacent operating room, the surgeon[6] excised a portion of the fat pad containing the donor's left hypogastric lymph node chain, and immersed the specimen in Ringer's solution. A total of 16 symmetrically placed, 2.0 to 2.5-cm. subcutaneous incisions had meanwhile been made in the inner aspect of the recipient's thighs (Figure 1), and packed with cotton-gauze sponges soaked in Ringer's solution. To minimize tissue trauma, hemostats and ligatures had been used sparingly.

Each of nine small lymph nodes was dissected free of the fat pad, cleaned of traces of pericapsular fat, rapidly and sterilely weighed on a Roller-Smith automatic precision balance, and placed separately in individual screw-cap vials, each vial containing 1.5 ml. of the recipient's serum to which penicillin and dihydrostreptomycin had been added to a concentration of 100 μg. per ml.

The wet weights of the nodes (in mg.) were respectively: 36.9, 41.8, 64.1, 110.1, 120.2, 127.8, 130.1, 213.8, and 82.5. The 82.5-mg. node was divided in halves and grossly examined. One half was placed in alcohol-formalin and the other frozen and saved.

Working rapidly, the operator transplanted each node in turn as follows: with a minimum of trauma, each node was sliced into four strips, each strip no greater than 2 mm. thick. Each strip was briefly checked for gross pathology, and then two strips were placed in an incision in the left thigh and two in the symmetrical incision in the right. No further chemotherapy was given the recipient, and no wound infections occurred. Healing progressed uneventfully, and at no time did sloughing occur.

Antigenic challenge of recipient and of donor. Seven days after transplantation, the recipient was challenged with 0.5 ml. of the previously described typhoid vaccine injected subcutaneously in the left arm. A booster dose of 0.5 ml. was given seven days later, and another, twenty days later.

To avoid problems of interpretation of titers in the donor, however, initial 0.5 ml. challenge was delayed until the ninth day, when the postoperative phase of heightened adrenal cortical activity had presumably passed. Booster doses of 0.5 ml. were given seven and, through an oversight, eighteen (rather than twenty) days later.

Excision biopsies. Nineteen days after transplantation, the strips from the node originally weighing 110.1 mg. were excised from their two symmetrical sites. At the same time, a right supraclavicular node was excised from

[4] Patient referred for study by Stuart O. Foster, M.D., Washington, D. C.

[5] Poliomyelitis Immune Globulin, Squibb and Sons, Lot No. 252-2, kindly supplied by the American National Red Cross.

[6] J. Keith Cromer, Department of Gynecology and Obstetrics, George Washington University School of Medicine, whose cooperation the authors gratefully acknowledge.

the recipient. Half of each of the specimens was fixed in alcohol-formalin; the remainder was subdivided grossly into node fragments, fat, and skin and was frozen and saved.

RESULTS

Immune responses

Tuberculin tests of the recipient two days after transplantation disclosed a powerful passively transferred delayed cutaneous hypersensitivity to tuberculin (Figure 2). Tuberculin reactivity then steadily increased to a plateau which extended from the 68th day to the 149th at a level equivalent to a reaction of 25 × 25 mm. induration to P.P.D. No. 1. Between the 149th and the 217th days reactivity fell off to a plateau at a lower, though still highly reactive, level. Reactivity was essentially unaffected by splenectomy on the 257th day.

In response to early challenges with typhoid vaccine, administered at sites distant from the transplants, the recipient developed low titers of typhoid H- and O-agglutinins (Figure 2) in a manner qualitatively similar to the higher titers observed in the donor (Figure 3). A booster challenge on the 98th day again elicited a secondary response, after which titers began to decline more rapidly. A final challenge on the 168th day elicited no response; instead, measurable H-agglutinins declined exponentially over the next 80 days with a slope statistically indistinguishable from that of passively infused H-agglutinins in other hypogammaglobulinemic patients (described below).

Biopsies

The donor's ninth hypogastric lymph node, which had not been transplanted, was histologically normal and quiescent and contained occasional plasma cells; a saline extract of the node was devoid of typhoid H- and O-antibody ($< 1:20$).

The recipient's left and right supraclavicular nodes, excised before and 19 days after transplantation, were virtually identical; both contained a total of only one or two dubious plasma cells and no antibody ($< 1:20$). Both contained abundant

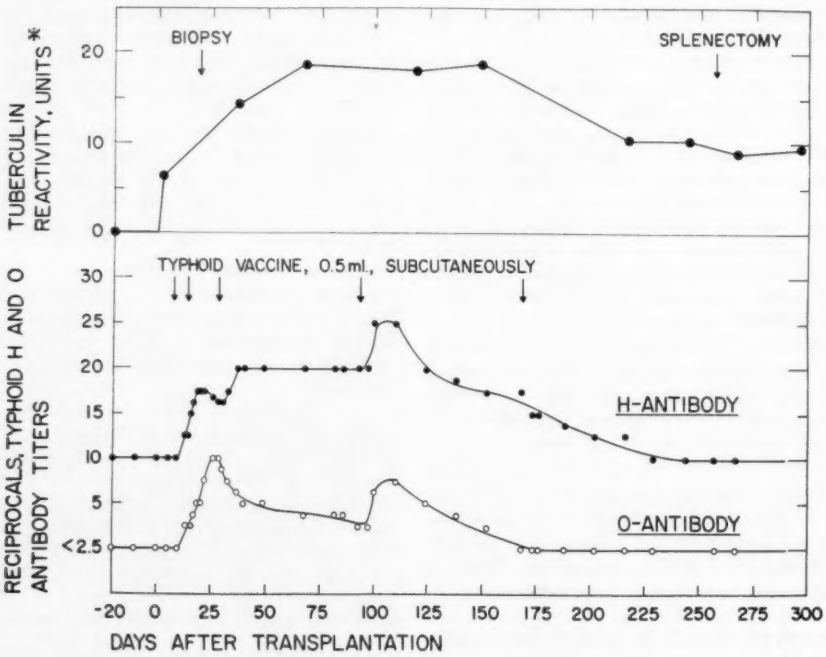

FIG. 2. IMMUNE REACTIONS OF TRANSPLANTED LYMPH NODES

*Tuberculin reactivity units: Millimeters mean maximum induration per log micro-microgram P.P.D. Each point represents the average of responses to two dilutions.

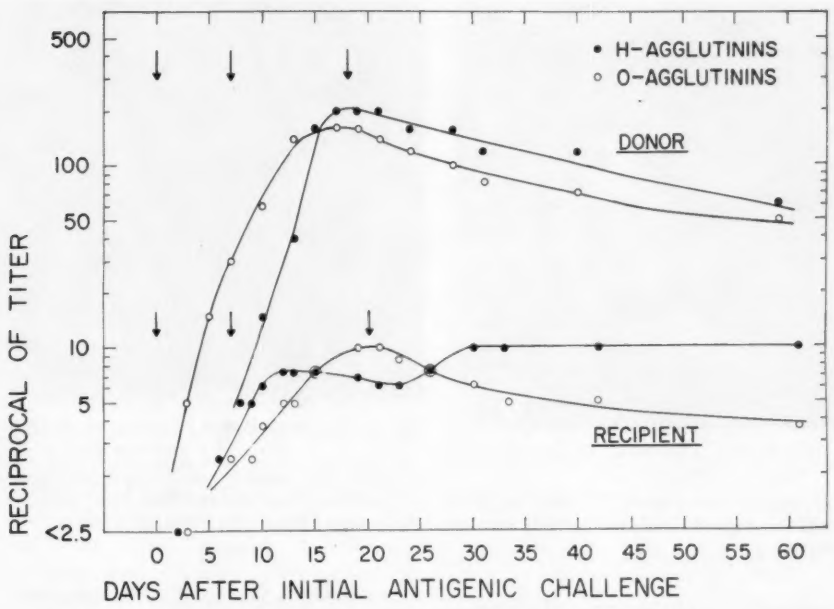

FIG. 3. DONOR AND RECIPIENT ANTIBODY TITERS AFTER TYPHOID IMMUNIZATION
The H-agglutinin titers observed in the recipient (Figure 2) are here corrected for a constant exogenous titer of 1 : 10.

lymphocytes and reticulum cells, but sparse and poorly developed germinal centers.

The 19th day excision biopsy of one of the transplanted nodes disclosed evidence of an initial take; the tissue contained moderate numbers of plasma cells and abundant tissue antibody. Extracts of various fragments grossly identified as node tissue (the bulk of each of which, however, was granulation tissue) titered 1 : 40, 1 : 40, 1 : 80, and 1 : 80 against H-antigen, and 1 : 20, 1 : 40, 1 : 80, and 1 : 200 against O-antigen. Extracts of adjacent specimens of fat and of the overlying skin and scar contained no demonstrable antibody. Serum H and O titers at the time of biopsy were 1 : 17.5 and 1 : 5, respectively.

Microscopically the transplant (Figure 4) contained architecturally disorganized cords of lymphoid tissue intimately penetrated by granulation tissue, with occasional foreign-body giant cells at the periphery. Higher magnification (Figure 5) disclosed intensely vascularized cords and strands of reticulum cells, lymphocytes, and scattered (but not abundant) plasma cells. Study of the giant cells by polarized light suggested that they were not reacting specifically to the donor tissue; each contained a bit of refractile cotton fiber, presumably from the surgical sponges used at the time of transplantation.

Therapeutic effects of transplantation

Despite a severe neutropenia (white blood counts 1200 to 2400, with 2 to 18 per cent neutrophils) the recipient remained free of major infections throughout the life of the transplant. During that interval there occurred episodes of oral moniliasis from the 76th day to the 81st and from the 130th to the 134th; a syndrome of fever, pharyngitis, cervical lymphadenopathy, and atypical lymphocytosis from the 86th day to the 96th; and facial furuncles from the 106th day to the 110th.

In contrast, the 100-day period following the presumed death of the transplant was filled with a succession of increasingly severe infections—ulcerative pharyngitis and stomatitis, recurrent oral moniliasis, facial furuncles, severe acute pansinusitis due to *Staphylococcus aureus,* and mul-

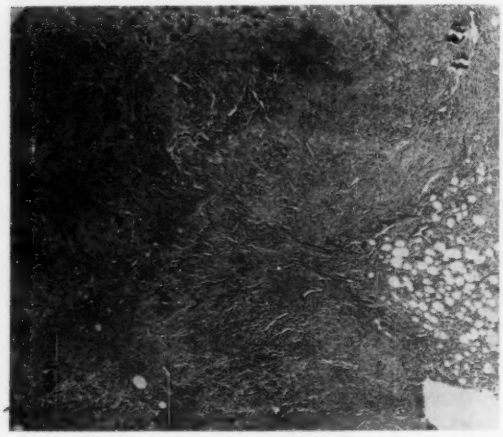

FIG. 4. EXCISION BIOPSY, TRANSPLANTED LYMPH NODE SLICE, NINETEENTH DAY

From left to right are seen granulation tissue, scattered giant cells, cords of lymphoid tissue, and partially necrotic fat (H & E × 85).

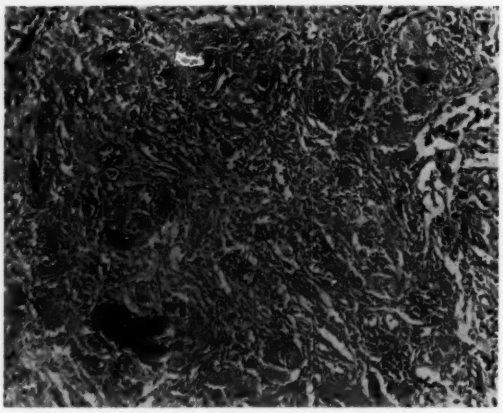

FIG. 5. EXCISION BIOPSY, TRANSPLANTED LYMPH NODE SLICE, NINETEENTH DAY

Within the intensely vascularized, disorganized cords of lymphoid tissue are reticulum cells, plasma cells, and foreign-body giant cells (H & E × 150).

tiple subcutaneous staphylococcal abscesses—which, together with the recipient's first severe thrombocytopenic episode, culminated in splenectomy on the 257th day. Postoperatively there occurred a prompt, sustained hematologic and symptomatic remission.[7]

Also in contrast, during a comparable, non-neutropenic 96-day period before transplantation there occurred only a single mild upper respiratory infection, lasting five days (17).

Neither during the life of the transplant nor after splenectomy did there occur any detectable rise in the recipient's serum gamma-globulin level.

In an attempt to make therapeutic use of the transplant, the patient was immunized, at bi-weekly intervals beginning on the 68th day, with standard parenteral doses of the following antigens: diphtheria toxoid-tetanus toxoid-pertussis vaccine, polyvalent influenza vaccine, and two doses of a combined respiratory bacterial vaccine.[8] In addition, four doses of an autogenous, formalinized *Escherichia coli* (communis) vaccine, prepared from the strain isolated from the recipient during a pyelonephritis and bacteremia fourteen months previously (17) and still present in the urine, were given during this period.

Although there was suggestive evidence of weak responses to several of the administered antigens, extensive titrations of pre- and post-challenge sera failed to demonstrate unequivocal antibody formation.

Immune interactions of transplant and recipient

To explore the possibility that the transplant might be rejecting the recipient, as well as *vice versa,* attempts were made to demonstrate isoimmune phenomena in several systems. Neither clinical nor laboratory evidence of nephritis, encephalitis, or collagen diseases appeared, and no evidence was adduced to implicate the transplanted nodes in the persistence of the recipient's neutropenia. The results of repeated Coombs tests were negative, and the recipient's first episode of thrombocytopenia occurred three months after transplant function had ceased.

[7] Multiple histological sections of the 630-gm. spleen revealed increased numbers of Malpighian follicles, prominent germinal centers, normal numbers of reticulum cells and lymphocytes, and prominent erythrocytophagocytosis by monocytes. Only a very few cells resembling plasma cells were seen, and there was no evidence of lymphoma, granuloma formation, amyloidosis, or collagen disease. A specimen of the liver was histologically normal. Cultures and animal inoculations of organ suspensions were negative for bacteria, mycobacteria, fungi, and *Toxoplasma.*

[8] Immunovac Respiratory Vaccine®, Parke Davis & Co.

Despite the challenge of small numbers of incompatible (Type A) red cells, the recipient failed to develop anti-A isoagglutinins in 1 : 1 dilution, measured by both the saline and the indirect Coombs techniques.

By the use of viable donor leukocytes as a slide agglutination antigen, it was demonstrated that the recipient failed to develop circulating leukocyte agglutinins in 1 : 1 dilution at any time after transplantation.

Skin tests, however, 250 and 266 days after transplantation, demonstrated a reproducible, specific, delayed, tuberculin-like cutaneous hypersensitivity to viable donor leukocytes. Intradermal injection of the leukocytes derived from 10 ml. of fresh donor blood elicited a reaction beginning at three hours and reaching a maximum diameter at 24 to 36 hours of 14 to 16 mm. of erythema and 10 to 12 mm. of tender induration, with a central hemorrhagic spot. Only transient, 3 to 4 mm. diameter erythematous responses were elicited by control injections of approximately equal numbers of leukocytes derived from the recipient and from normal Type O, tuberculin positive; Type B, tuberculin negative; and Type A, tuberculin negative persons, as well as of donor plasma containing dextrose-dextran-sequestrene.[9]

Immunochemical studies

The results of immunochemical studies (Figures 6, 7) indicate that under the conditions of biological assay described above 1 unit of typhoid H-agglutinating activity synthesized by the specific donor (or donor tissue) is equivalent to .126 ± .009 microgram gamma-globulin N per ml. serum and that 1 unit of similarly specific typhoid O-agglutinating activity is equivalent to .102 ± .026 microgram $beta_2$-globulin N per ml. serum. With the whole bacilli antigens employed, the ratios in equivalence-point precipitates of antibody N : antigen N for the H and O systems were, respectively, 1 : 2.85 and 1 : 5.27.

Estimation of rates of antibody synthesis

In the absence of demonstrable recipient antibody synthesis and with the knowledge that extracts only of the transplants contained large amounts of antibody, it was concluded that all observed titers represented antibody synthesized by the transplant. A general equation for calculating daily total synthesis of typhoid antibody by the transplant was therefore formulated as follows:

If S be the weight of H- or O-antibody synthesized and released per gram of lymph node tissue per day; I, the daily increment in serum titer in agglutinating units; D, the units of antibody passively degraded per day; V, the apparent body fluid volume or compartment in which the antibody is diluted; K, the constant that relates units of agglutinating activity to weight of antibody nitrogen; 6.2, the protein : nitrogen ratio in both gamma- and $beta_2$-globulin (18); and W, the weight in grams of transplanted lymph node tissue, then on any given day

$$S = \frac{(I + D)(V)(K)(6.2)}{W}. \qquad (1)$$

Values of I were read from the curve of antibody titer derived from graphic analysis of the observed titers (Figure 3).

Values of D were calculated by substituting each day's average serum titer for C in the equation describing one day's exponential decline of passively acquired antibody from a level of C to a level of C_0,

$$\frac{\text{Log } C - \text{Log } C_0}{1} = \text{Slope} = \text{Constant}, \qquad (2)$$

solving for C_0 and subtracting from C. The appropriate constant for H-antibody was determined, and that for O-antibody was assumed, as follows:

H-antibody: After administration of 50 gm. of human gamma globulin [5] to two other hypogammaglobulinemic patients, the passive rates of decline of serum gamma globulin and of six common antibodies were determined by *in vitro* assays of multiple periodic serum specimens drawn over a 63- to 70-day period (19). The half-life of typhoid H-agglutinin, so determined, was 35.0 ± 3.6 days, corresponding to a slope of 0.0086.

O-antibody: Although the half-life of $beta_2$-globulin in human beings has never been determined, sparse indirect evidence suggests that $beta_2$-globulins are passively degraded considerably faster than gamma globulins. The equations with

[9] Because of the possibility of immunizing the recipient against her prospective donor, the recipient was not skin-tested with donor leukocytes before transplantation.

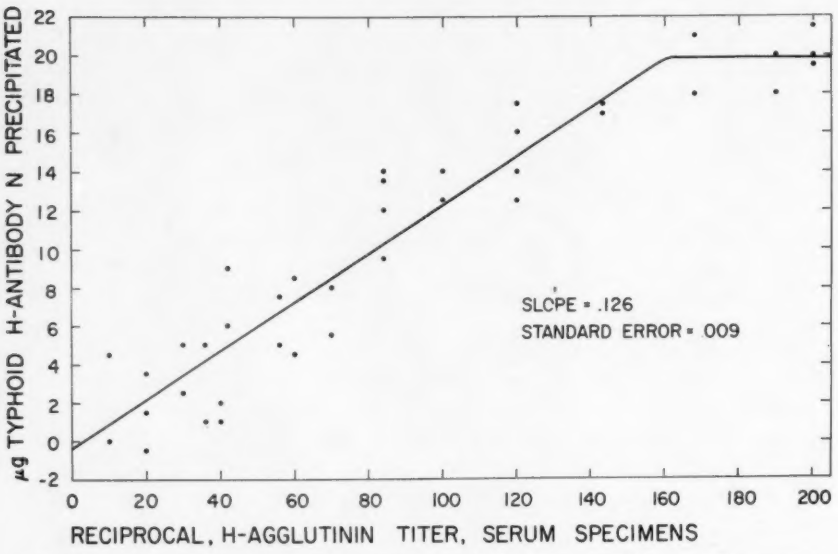

FIG. 6. ANTIBODY NITROGEN PRECIPITATED WITH 56.5 MICROGRAMS TYPHOID H-ANTIGEN NITROGEN

which Wiener demonstrated that newborns passively degrade diphtheria antitoxin at a rate identical with that of adults (20), when applied to the data of Smith on the decline of passively acquired compatible isoagglutinins in newborns (21), indicate that these $beta_2$-globulins have a half-life of 12 to 18 days. Biosynthetic determinations of the half-lives of heterogeneous plasma protein fractions, of which $beta_2$-globulins were only one of many components, have yielded values of 6.2 to 7.8 days for fractions I + III (22) prepared by Cohn Method 10 (23), and 17 days for pooled $alpha_1$-$alpha_2$-beta-globulins (24).

After two transfusions of fresh Type B, Rh positive blood, the recipient's passively acquired anti-A isoagglutinin titers were followed for 28

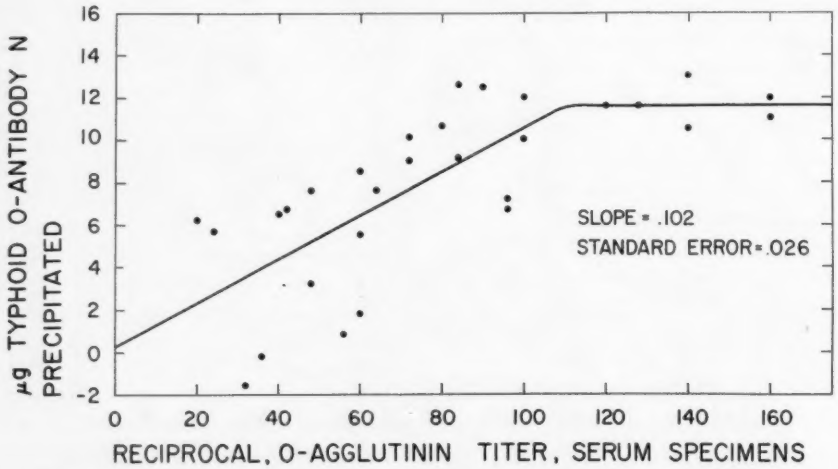

FIG. 7. ANTIBODY NITROGEN PRECIPITATED WITH 61.1 MICROGRAMS TYPHOID O-ANTIGEN NITROGEN

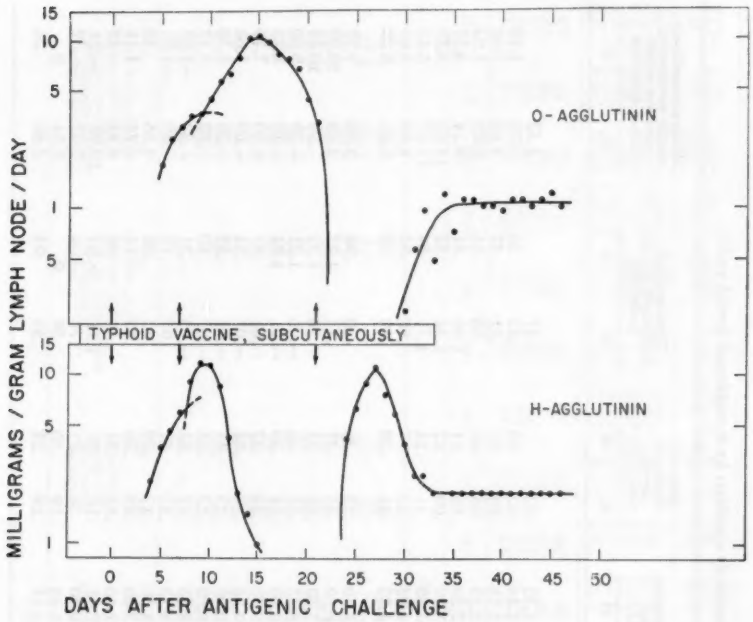

FIG. 8. ANTIBODY PROTEIN SYNTHESIS BY TRANSPLANTED LYMPH NODES

days. Analysis of seven determinations yielded a linear regression curve with a half-life of 16.7 days and a standard error of 2.5 days.

Employing these data and noting that the maximum slopes in the declining phase of the curves of O-antibody titer in both donor and recipient corresponded to half-lives of 15 ± 2 days (Figures 2 and 3), a 15-day half-life of O-agglutinins (or slope of 0.020) was assumed.

Compartment size: Previous studies of the recipient had demonstrated that after equilibration administered gamma globulin (including typhoid H-antibody) behaves as if diluted in a fluid volume equivalent to 20 per cent of body weight (17), *i.e.*, 25 to 30 per cent is found in the plasma, and the remainder, in extravascular compartments. In this study, in which the recipient's weight (47 Kg.) remained essentially constant throughout the period of observation, the volume of distribution V of typhoid H-antibody was therefore equivalent to 9.4 liters.

It was not possible to determine directly V for typhoid O-antibody, since no safe concentrated source of human O-antibody is available for human administration. As a near approximation, the V for anti-A isoagglutinins, which are chemically and electrophoretically similar to typhoid O-agglutinins, was determined. After transfusion of the recipient with fresh blood containing compatible plasma of known isoagglutinin content, anti-A isoagglutinins behaved as if diluted in a volume equivalent to 20 ± 3 per cent of body weight (25). Since this figure is compatible with the known presence of typhoid O-antibody and isoagglutinins in numerous tissues and interstitial fluids (26–29), it appeared reasonable to assume V for typhoid O-antibody to be identical with that of H-antibody.

Employing these data and assuming, further, prompt (24-hour) mixing of newly synthesized antibody and full survival of the transplant (W = 844.8 mg. before excision biopsy and 734.7 after), minimum rates of antibody synthesis were calculated (Table I) and graphed (Figure 8).

DISCUSSION

Clinical observations

The present study confirms the demonstration by Good and Varco (1–3) of the impaired ability of the hypogammaglobulinemic patient to respond

TABLE I

Serum titrations and estimated daily rates of typhoid H- and O-antibody synthesis by transplanted lymph nodes

Days after initial antigenic challenge	Reciprocals of four serum titrations (*units/ml.*)		Reciprocal mean observed endogenous serum titer (*units/ml.*)		Reciprocal estimated graphic titer (*units/ml.*)		Antibody destroyed per day (*units/ml.*)		Total antibody synthesized per day (*units/ml.*)		Milligrams antibody protein synthesized/ gram wet weight lymph node/day	
	H	O	H	O	H	O	H	O	H	O	H	O
2	10, 10, 10, 10		0									
3	10, 10, 10, 10	< 2.5 × 4	0	<2.5	1.0							
4					1.25	1.25	.02		.27		2.32	
5		< 2.5 × 4		<2.5	1.65	1.45	.03	.06	.43	.26	3.70	1.79
6	10, 10, 15, 15		2.5		2.15	1.75	.04	.07	.54	.37	4.64	2.55
7	10, 10, 15, 15	2.5, 2.5, 2.5, 2.5	2.5	2.5	2.80	2.10	.05	.09	.70	.44	6.02	3.03
8					3.80	2.50	.06	.11	1.06	.51	9.14	3.52
9	15, 15, 15, 15	2.5, 2.5, 2.5, 2.5	5.0	2.5	5.05	2.90	.09	.12	1.34	.52	11.52	3.59
10	15, 15, 15, 20	2.5, 3.75, 3.75, 5	6.25	3.75	6.25	3.40	.11	.14	1.31	.64	11.27	4.42
11					7.10	4.05	.13	.17	.98	.82	8.43	5.65
12	15, 15, 20, 20	5, 5, 5, 5	7.5	5.0	7.45	4.75	.14	.20	.49	.90	4.21	6.20
Biopsy												
13	15, 15, 20, 20	5, 5, 5, 5	7.5	5.0	7.50	5.50	.15	.23	.20	.98	1.98	7.77
14					7.50	6.50	.15	.27	.15	1.27	1.48	10.07
15	15, 15, 20, 20	5, 7.5, 7.5, 10	7.5	7.5	7.45	7.50	.15	.32	.10	1.32	.99	10.46
16					7.25	8.35	.14	.36	−.06	1.21	−.59	9.60
17					7.10	9.00	.14	.39	−.01	1.04	−.10	8.25
18					6.95	9.55	.14	.42	−.01	.97	−.10	7.70
19	7.5, 20, 20, 20	10, 10, 10, 10	6.88	10	6.75	9.95	.13	.44	−.07	.84	−.69	6.66
20					6.60	10.05	.13	.45	−.02	.55	−.20	4.36
21	15, 15, 15, 20	10, 10, 10, 10	6.25	10	6.40	10.00	.13	.45	−.07	.40	−.69	3.17
22	15, 15, 15, 20		6.25		6.25	9.35	.12	.44	−.03	−.21	−.30	−1.67
23		7.5, 7.5, 10, 10		8.75	6.20	8.75	.12	.41	.07	−.19	.69	−1.51
24					6.50	8.30	.12	.39	.42	.06	4.16	.48
25					7.00	7.80	.13	.36	.63	−.14	6.24	−1.11
26	15, 15, 20, 20	7.5 × 4	7.5	7.5	7.75	7.40	.14	.34	.89	−.06	8.82	−.48
27					8.70	7.05	.16	.32	1.11	−.03	10.98	−.24
28					9.30	6.70	.18	.31	.78	−.04	7.72	−.32
29					9.70	6.40	.19	.30	.59	0	5.84	0
30	20, 20, 20, 20	5, 5, 7.5, 7.5	10	6.25	9.90	6.15	.19	.28	.39	.03	3.86	.24

TABLE I—*Continued*

Days after initial antigenic challenge	Reciprocals of four serum titrations (*units/ml.*)		Reciprocal mean observed endogenous serum titer (*units/ml.*)		Reciprocal estimated graphic titer (*units/ml.*)		Antibody destroyed per day (*units/ml.*)		Total antibody synthesized per day (*units/ml.*)		Milligrams antibody protein synthesized/gram wet weight lymph node/day	
	H	O	H	O	H	O	H	O	H	O	H	O
31					9.95	5.95	.20	.27	.25	.07	2.48	.56
32					10.0	5.80	.20	.27	.25	.12	2.48	.95
33	20, 20, 20, 20	5, 5, 5, 5	10	5.0	10.0	5.60	.20	.26	.20	.06	1.98	.48
34					10.0	5.50	.20	.25	.20	.15	1.98	1.19
35					10.0	5.35	.20	.24	.20	.09	1.98	.71
36					10.0	5.25	.20	.24	.20	.14	1.98	1.11
37					10.0	5.15	.20	.24	.20	.14	1.98	1.11
38					10.0	5.05	.20	.23	.20	.13	1.98	1.03
39					10.0	4.95	.20	.23	.20	.13	1.98	1.03
40					10.0	4.85	.20	.22	.20	.12	1.98	.95
41					10.0	4.77	.20	.22	.20	.14	1.98	1.11
42	20, 20, 20, 20	5, 5, 5, 5	10	5.0	10.0	4.70	.20	.21	.20	.14	1.98	1.11

Additional serum titrations

Days after initial antig. challenge		61	75	79	86	90	93	103	117	131	145	161	166	169	181	195	209	222	237
Reciprocals, four serum titrations (*units/ml.*)	H	10	20	20	20	20	20	20	20	15	15	15	15	15	10	10	10	10	10
		20	20	20	20	20	20	20	20	20	15	15	15	15	15	10	10	10	10
		20	20	20	20	20	30	30	20	20	20	20	15	15	15	15	15	10	10
		30	20	20	20	20	30	30	20	20	20	20	15	15	15	15	15	10	10
	O	3.75	3.75	2.5	2.5	2.5	7.5	5	5	3.75	2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
		3.75	3.75	3.75	2.5	2.5	7.5	7.5	5	3.75	2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
		3.75	3.75	3.75	2.5	2.5	7.5	7.5	5	3.75	2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
		3.75	3.75	5	2.5	2.5	7.5	10	5	3.75	2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
Recip. mean endog. titer (*units/ml.*)	H	10	10	10	10	10	15	15	10	8.75	7.5	7.5	5	5	3.75	2.5	2.5	0	0
	O	3.75	3.75	3.75	2.5	2.5	7.5	7.5	5	3.75	2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5

to foreign tissue antigens and of the feasibility of artificially endowing him for a prolonged period with a functional miniature reticulo-endothelial system. The data suggest that the eight transplanted lymph nodes survived with full function for 100 to 110 days, and that destruction of the tissue occurred slowly, culminating in total unreactivity about the 160th day.

The recipient's passively acquired tuberculin hypersensitivity, which reached a peak only gradually, remained constant for three months, and then, coincident with cessation of node function, fell abruptly to a lower level, has continued at that level for a prolonged period. In the absence of evidence for transmission of tuberculosis, this pattern of transferred reactivity conforms to one of those described by Lawrence (15) and interpreted by him and by Chase (30) as suggestive either of two distinct phases—one passive and one "active" —in the recipient's handling of a single transfer substance in intact or disrupted sensitized leukocytes or, alternatively, of two leukocyte substances —one more and one less available—involved in the transfer. If it can be assumed that the transplant was totally destroyed by the 160th day, then the evidence tends to support the first hypothesis, inasmuch as only a portion of the reactivity was lost when the exogenous leukocyte source of hypersensitivity was removed.

The recipient's development of a specific, delayed-type cutaneous hypersensitivity to leukocytes derived from the donor, together with her failure to develop circulating agglutinins to these leukocytes, are in accord with abundant evidence of the pre-eminent role of fixed tissue antibody rather than of circulating antibody mechanisms in the rejection of homografts and homotransplants (31–34). It was not deemed clinically justifiable to test the possibility that the recipient would manifest an accelerated rejection of another lymph node from the specific donor.

All other technical considerations being equal, the limiting factor in the longevity of transplants to patients with hypogammaglobulinemia is thus apparently the degree to which this diffuse disease of the reticulum has impaired the patient's capacity to develop fixed tissue antibody. Though usually markedly impaired, this capacity is well retained in a minority of patients with acquired hypogammaglobulinemia (35); moreover, its presence—though very weak—in congenitally hypogammaglobulinemic children has been demonstrated by Good (3) and Porter (36).

The histological findings in the excision biopsy neither confirm nor deny the evidence for either the plasma cell or the lymphocyte as the major cellular source of circulating antibody (37). The finding of relatively small numbers of plasma cells in microscopic sections may mean only that the pathologist's "half" of the specimen contained a small, unrepresentative portion of the transplant.

Whether or not the transplant ameliorated the recipient's hypogammaglobulinemia is a moot point; her clinical course before, during and after transplantation differed in no striking way from that of progressively severe hypersplenism, or of progressive neutropenia of any etiology. Conversely, it may be that the presence of neutropenia during the life of the transplant precluded effective utilization of any antibody synthesized by the transplant and that transplantation was therefore not given a fair test. Whether the theoretically beneficial effects of lymph node transplantation justify the risks (principally that of contracting serum hepatitis) is thus still conjectural, at least in the case of acquired hypogammaglobulinemia. In congenital hypogammaglobulinemia, on the other hand, in which one may anticipate survival of transplants for years rather than months, lymphoid tissue homotransplantation remains an attractive therapeutic possibility (35).

Laboratory observations

As predicted by Boyd and Hooker (38), who demonstrated an inverse log-log linear relationship between the ratio by weight of antibody to antigen in equivalence-point precipitates and the molecular weight of antigen, the equivalence-point ratios of typhoid H- and O-antibodies to whole bacilli antigens were very low. Consequently, individual measurements of antibody precipitated from most serum specimens fell close to the limits of accuracy of the quantitative agglutinin procedure (9)—just beyond the error of the micro-Kjeldahl technique —resulting in curves the standard error of the slopes of which is relatively high: 7 per cent for H-antibody and 25 per cent for O-antibody.[10]

[10] Because comparably high standard deviations were associated with both repeated determinations of the antibody nitrogen in one serum and single determinations of

TABLE II

Measured rates of protein synthesis by various human and animal tissues, and rates estimated from recent human biosynthetic turnover data

Tissue	Reference	Method	Protein synthesized	Experimental conditions	Rate of synthesis (*mg./gm. wet weight tissue/day*)
			Normal human tissues, *in vivo*		
Lymph node	This paper	Tissue slices homotransplanted to hypogammaglobulinemic adult	Typhoid O-beta$_2$-globulin	Immune response: Peak, primary response	3.6
				Peak, secondary response	10.5
				Artificial acquired immunity, late	1.0
			Typhoid H-gamma-globulin	Peak, primary response	6.0
				Peak, secondary response	11.0, 11.5
				Artificial acquired immunity, late	2.0
Reticulo-endothelial system	(22)	Oral S^{35} and S^{35} labeled, i.v.	Gamma-globulins	Steady state*	1.7–3.9
	(24)	Oral S^{35}			1.7–5.4
	(51)	Oral N^{15}			5.4–5.8
Liver	(22)	Oral and i.v. S^{35} and S^{35} labeled, i.v.	Total plasma protein	Steady state†	7–15
	(53)‡				
	(22)	Oral S^{35} and S^{35} labeled, i.v.			3.4–6.4
	(24)	Oral S^{35}	Albumin	Steady state†	2.9–5.3
	(51)	Oral N^{15}			7–10
	(22)	Oral S^{35} and S^{35} labeled, i.v.	Fibrinogen	Steady state†	1.4–2.4
			Normal animal tissues, *in vitro*		
Liver (rat)	(55)	Perfused whole liver	Total plasma protein	Optimal perfusion conditions; tissue from fasting rats	15–18
	(56)			Tissue from non-fasting rats	50–70§
Liver (chicken)	(58)	Tissue slices	Albumin	Optimal incubation conditions	2.5–3.0
Pancreas (pigeon)	(59)	Tissue slices	Amylase	Tissue depleted of zymogen by carbamyl-choline pretreatment	15–25
Anterior pituitary (rat)	(60)	Tissue slices	Protein hormones	Optimal incubation conditions	45–55

* Estimates assume average adult exchangeable gamma-globulin pool of 75 gm. (22), and average weight of total adult reticulo-endothelial tissue of 500 gm. (52).

† Estimates assume average adult exchangeable total plasma protein pool of 530 gm., albumin of 300 gm., and fibrinogen of 20 gm. (54), and average weight of adult liver of 1500 gm.

‡ Serum protein.

§ Steinbock and Tarver estimate rate of 45 to 65 mg. per gm. wet weight liver per day in intact rat (57).

Although it is not possible to state categorically that the donor and the transplanted nodes underwent a primary immune response to typhoid vaccine, the data suggest that they did. Particularly with respect to H-antibody—ordinarily the most prominent antibody developed in response to immunization—the relatively long initial induction phase in the donor and in the recipient and the

different sera of equal titer (Table IV), it was concluded that the major error in the experiment lay in the immunochemical method rather than in the agglutinin titrations.

relatively low peak titers achieved in the donor (Figure 3) more closely resemble the pattern of the primary than of the secondary immune response to typhoid vaccine (39, 40).

Many investigators have demonstrated and have roughly measured the ability of specifically sensitized human and animal reticulo-endothelial tissue to muster a secondary immune response when brought in contact with the sensitizing antigen in a new normal or irradiated homologous or heterologous host or in the test tube (37, 41, 42). In contrast, because of the lack of an animal counterpart to the syndrome of hypogammaglobulinemia, technical problems associated with inducing varying degrees of immunologic unresponsiveness in animals through barely sub-lethal doses of X-ray or reticulo-endothelial blocking agents (40, 43, 44), and the nature of immunization itself, semiquantitative studies of the entire immune response, including the primary phase, have been accomplished in the intact host only with difficulty (45) and *in vitro,* not at all (40, 46).

The rates of antibody protein synthesis found in the present study (Table I) clearly represent first attempts at reasonable estimates of the order of magnitude of the immune response in man, rather than precise measurements of the reaction. Our estimates are, first, conditioned by the age of the donor, the metabolic state of the recipient, and the relative technical success of the transplantation procedure; second, subject to the cumulative error of the immunochemical measurements, the graphic titers, and the passive half-life values; and third, closely tied to our most critical assumptions and estimates—those concerning pool size and equilibration time. As has been recently emphasized (47, 48), the paucity of information on the last two points constitutes the chief stumbling-block to accurate studies of the biosynthesis and turnover of labeled proteins. Estimates of equilibration time of various plasma proteins range from 4 hours to 6 days (29, 48).

Pool size studies in this laboratory on three other hypogammaglobulinemic patients (19) confirm the results of Gitlin and Janeway (49), who demonstrated that as in the case of albumin (50) approximately half the gamma-globulin pool is intravascular, and the other half extravascular, corresponding to an apparent volume of distribution of 10 to 12 per cent of body weight. The excessively large (20 per cent) pool size in the recipient in the present study presumably reflects expanded plasma and extracellular fluid volumes, caused by her anemia (hematocrit 26 to 28) and moderate congestive heart failure.

With these reservations, the results of the present study are listed with those of several recent studies of protein synthesis in man and in animals (Table II). The comparison emphasizes the striking flexibility and relatively enormous proteosynthetic capacity of human lymphoid tissue. In response to a moderate antigenic stimulus, and with little or no previous experience, human lymphoid tissue is capable of synthesizing highly

TABLE III

Titration of ten sera of known relative typhoid O-agglutinin titer *

Known relative titer (*units/ml.*)	Two-fold plus intermediate dilutions						Two-fold dilutions					
	Four determinations of titer				Observed mean titer (*units/ml.*)	Error, mean titer (*per cent*)	Four determinations of titer				Observed mean titer (*units/ml.*)	Error, mean titer (*per cent*)
160	160	160	160	160	160	0	160	160	160	160	160	0
152	160	160	160	160	160	5	160	160	160	160	160	5
144	160	160	160	160	160	11	160	160	160	160	160	11
136	160	120	160	160	150	10.5	160	160	160	160	160	18
128	160	120	120	160	140	9.5	160	80	160	160	140	9.5
120	120	120	120	160	130	8.25	160	80	160	160	140	16.5
112	120	120	120	120	120	7	80	160	160	160	140	25
104	120	120	120	120	120	15	80	80	80	80	80	23
96	120	80	120	120	110	14.5	80	80	80	80	80	17
88	120	80	80	60	90	2.25	80	80	40	80	70	20.5
Mean error, mean titers, per cent						8.3						14.6
Standard deviation, individual determinations, per cent						12.9						23.8

* Mixtures in varying proportion of a post-typhoid immunization donor serum and known negative hypogammaglobulinemic serum, made up to encompass one dilution step, in decrements of 5 per cent. Mixtures were coded, scrambled, and titered by the method described.

TABLE IV

Immunochemical estimation of typhoid O-agglutinins: comparison of six determinations of one serum and single determinations of six sera of equal titer *

	One serum						Six sera						Antigen, 1.0 ml.					
Optical density, ppt. N	.148	.141	.144	.140	.141	.153	.148	.139	.139	.144	.153	.151	.136	.132	.137	.133		
Ppt. N, μg.	68.7	65.4	66.8	65.0	65.4	71.0	68.7	64.5	64.5	66.8	71.0	70.1	63.1	61.2	63.6	61.7		
Agglutinin N, μg., by subtraction	7.1	3.8	5.2	3.4	3.8	9.4	7.1	2.9	2.9	5.2	9.4	8.5						
Agglutinin N:																		
Mean, μg.			5.5						6.0									
Stand. Dev., μg.			2.4 (44%)						2.8 (47%)									

* Volume of each serum specimen adjusted to contain 60 agglutinating units.

specific protein at a rate comparable with that at which endocrine and exocrine tissues synthesize such unvarying protein products as enzymes and hormones.

SUMMARY

After subcutaneous homotransplantation to a hypogammaglobulinemic adult, eight unsensitized human lymph nodes survived with full function for 100 to 110 days, and partial function for 50 to 60 additional days. Ultimate rejection by the recipient was associated with a delayed cutaneous hypersensitivity to leukocytes from the lymph node donor.

By the use of appropriate data on the immunochemistry, pool size, and passive half-life of typhoid antibodies, the immune response of the transplant was measured in terms of milligrams of antibody protein synthesized per gram wet weight of lymph node tissue per day.

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THE EFFECT OF LARGE DOSES OF DESICCATED THYROID ON THE DISTRIBUTION AND METABOLISM OF ALBUMIN-I^{131} IN EUTHYROID SUBJECTS

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It has been observed that I^{131} labeled human serum albumin, administered intravenously to normal man, is ultimately distributed between extravascular tissues and plasma in the approximate ratio of 60:40 (1, 2). This ratio is not appreciably altered in heart failure with edema (2), but may be increased somewhat in hepatic cirrhosis with ascites (1). One of the highest extravascular-intravascular ratios ever observed in this laboratory was in a patient with myxedema, in whom 73 per cent of the iodinated albumin remaining within the body at distribution equilibrium was in the extravascular spaces (2). The high extravascular albumin content in myxedema may be related to increased skin thickness since it has been shown that skin contains a disproportionately large fraction of the total extravascular albumin (3). It therefore seemed of interest to evaluate whether extravascular-intravascular shifts of serum albumin could be initiated in euthyroid subjects by the administration of large doses of thyroid hormone.

It had also been observed previously that albuminuric subjects do not generally compensate for renal losses by an increase in the rate of albumin synthesis but do so rather by a decrease in the rate of albumin catabolism (2). Because the possibility of hepatic damage in nephrotic subjects has been suggested (4), it also seemed of interest to evaluate whether subjects with normal liver function would respond with an increase in the rate of albumin synthesis to the increased rate of albumin degradation anticipated in response to excessive thyroid hormone stimulation.

METHODS

Nine male patients at the Veterans Administration Hospital, Bronx, New York, were subjects of this study. The illnesses responsible for hospitalization are given in Table I. There was no suspicion of thyroid disease nor detectable proteinuria in any of the patients. Seven subjects were maintained on a regular hospital diet, ad libitum, throughout the study. Two subjects (R. W. and S. K.) received protein supplements of 90 gm. per day in addition to the regular diet.

The distribution and metabolism of albumin were studied by means of I^{131} labeled human serum albumin. Following the intravenous administration of albumin-I^{131}, observations of plasma and urinary I^{131} were made for a control period of 15 to 22 days, following which, observations were continued for another 13 to 22 days while the subjects received 12 to 18 grains of desiccated thyroid, U.S.P., per day (Figure 1). For the purpose of reevaluating the distribution of albumin at the end of this period, a second dose of albumin-I^{131} was administered and observations were continued for another 9 to 14 days during continuing thyroid therapy except in patient D. C. in whom thyroid hormone was discontinued at the time of administration of the second dose of albumin-I^{131}.

The iodinated albumin used in these experiments was prepared according to methods previously described (1) and contained approximately one iodine atom per protein molecule. These preparations have previously been observed to undergo a uniform rate of metabolic degradation *in vivo* (1, 2). Since it has been shown that I^{131} labeled albumin of high specific activity may be damaged sufficiently by radiation to result in rapid *in vivo* degradation (5), the albumin-I^{131} employed in this study was prepared with relatively low specific activity (< 21.5 μc. per mg.). Each patient received a total of 150 to 250 μc. intravenously in 2 injections. Lugol's solution, 10 drops 3 times a day, was administered daily to block thyroidal uptake of I^{131}. Heparinized blood samples were taken without tourniquet stasis 15 minutes, 2 hours, and 6 hours after injection, and at almost daily intervals thereafter. Twenty-four-hour urine collections were obtained daily except in an occasional instance when a pooled weekend urine was collected. Plasma and urine samples were assayed for radioactivity in a well type scintillation counter with a sensitivity of 1.0×10^6 counts per min. per μc. I^{131} above a background of approximately 200 counts per min. Total serum protein concentration was determined by the Kjeldahl method and serum albumin concentration was determined by the method of Kingsley (6) at 3 to 7-day intervals throughout the period of study.

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TABLE I

Data on the distribution and metabolism of albumin before and during the administration of desiccated thyroid

Lot of albumin received	Subject	Diagnosis	A Plasma concentration (gm./100 ml.) Total protein Cont.*	Total protein Exp.†	B Albumin Cont.	Albumin Exp.	Plasma volume (ml.) Cont.	Plasma volume (ml.) Exp.	C Albumin degraded daily (gm.) Cont. P‡	Cont. U‡	Cont. Cl‡	Exp. Cl	D Duration of thyroid therapy (days)	E Albumin degraded in excess of similar control period (gm.)	F TEA§ (gm.) Cont.	TEA§ (gm.) Exp.	G TEA loss (gm.)	H (E–G) Net synthesis of albumin (gm.)	I Albumin partition (gm.) Intravascular Cont.	Intravascular Exp.	Extravascular Cont.	Extravascular Exp
I	E. A.	Psoriasis	7.46	6.11	4.59	4.18	3,320	3,770	17.0	18.0	17.0	18.9	17	32	345	335	10	22	148	158	197	177
I	D. C.	Convalescent Poliomyelitis	6.99	6.41	4.53	4.36	2,620	2,970	13.4	11.1	11.2	14.8	12	43	296	305	−9	52	119	129	177	176
	F. M.	Neuro-dermatitis	7.03	6.38	4.22	4.00	3,140	3,620	15.8	14.0	14.4	18.4	16	64	343	326	17	47	133	145	210	181
II	T. M.	Multiple Sclerosis	7.02	5.88	4.89	4.00	3,660	3,760	18.6	18.2	17.6	24.6	20	140	408	353	55	85	179	150	229	203
	L. U.	Traumatic Paraplegia	7.21	6.20	4.59	3.96	2,450	2,650	18.9	17.5	17.0	19.8	13	36	309	305	4	32	112	105	207	200
	L. Z.	Keratoconus	7.11	6.44	5.18	4.82	2,770	3,070	11.2	11.7	11.3	14.9	12	43	374	360	14	29	144	148	230	212
III	P. M.	Psoriasis	6.83	5.50	4.41	4.00	2,770	3,210	16.2	14.0	13.9	18.0	22	90	265	262	3	87	122	128	1 43	134
IV	R. W.	Amputee	6.78	6.70	4.22	3.90	3,290	3,450	18.6	16.8	16.6	22.8	19	118	355	335	20	98	139	135	216	200
	S. K.	Convalescent Poliomyelitis	6.89	6.75	4.91	4.50	3,880	4,130	20.2	21.5	18.1	26.8	19	165	446	409	37	128	209	186	237	221
Mean values			7.03 ±0.20 (S.D.)	6.26 ±0.37	4.62 ±0.31	4.19 ±0.29	3,080	3,400	16.5	15.8	15.2	19.8	16.6	81	349	332	17	64	145	143	205	189
Per cent change				−11.0		−9.3		+10.4				+30.3					−4.9			−1.6		−7.8

* Cont.—Before desiccated thyroid administration.

† Exp.—During desiccated thyroid administration.

‡P‡U‡Cl—From plasma concentration curve, daily urinary excretion, and "metabolic clearance" techniques, respectively (see text).

§ TEA–Total exchangeable albumin.

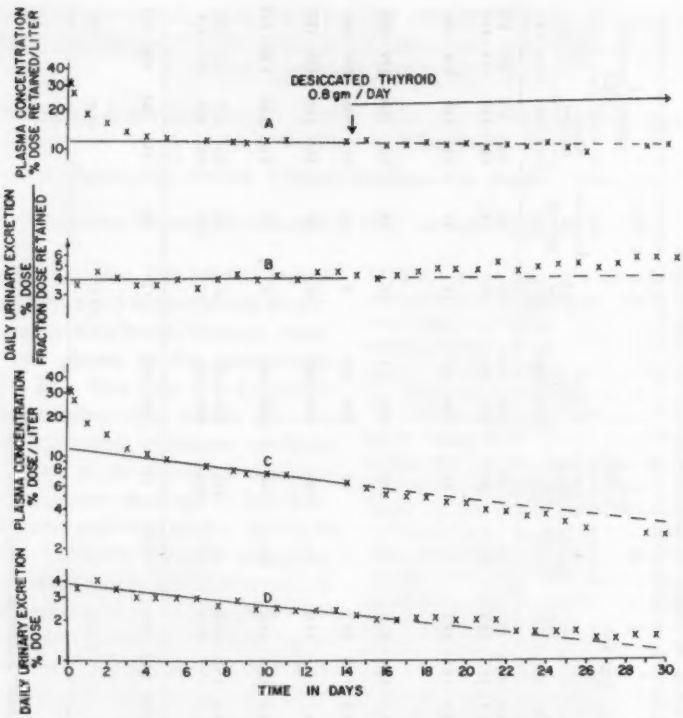

FIG. 1. TYPICAL SET OF CURVES FOR PLASMA AND URINE DATA (PATIENT F. M.) FOLLOWING ALBUMIN-I^{131} ADMINISTRATION (SEE TEXT)

Plasma volume and total exchangeable albumin (TEA) were determined by methods previously described (1).

The rate of metabolism of serum albumin was determined by several different means. Methods employing the rate constant of decrease in plasma concentration of albumin-I^{131} after distribution equilibrium or the rate of urinary excretion of I^{131} released by degradation of albumin-I^{131} have been previously described (1) and yielded essentially identical values. However, the validity of these methods depends upon the maintenance of steady state conditions. During the control period, serum albumin concentrations remained constant, and for practical purposes it may be assumed that steady state requirements were satisfied. Under these conditions the rate of synthesis is equal to the rate of degradation. However, during the experimental period of thyroid administration, changes in the distribution and the rate of degradation altered the steady state so that the validity of these methods is vitiated. Therefore, the following method, the validity of which is independent of the steady state, was employed for comparison of albumin degradation during control and experimental periods. Since radioactivity excreted in the urine in the absence of proteinuria represents I^{131} released by metabolic degradation of albumin-I^{131}, the amount degraded each day was calculated as the product of the apparent renal clearance of plasma I^{131} ("metabolic clearance") and the plasma concentration of albumin (Figure 2).[3] The total amount degraded over each period was then obtained from the sum of the daily values.[4] The quantity of albumin synthe-

[3] It has been shown previously that the urinary excretion of I^{131} reflects very closely the degradation of albumin-I^{131} owing to the very rapid rate of renal excretion of the I^{131} released by protein degradation compared to the rate of degradation itself (1). "Metabolic clearance" methods have also been used in the study of thyroid hormone degradation (7).

[4] In subject P. M. the second dose of albumin-I^{131} was administered 4 days after observations on the degradation of the first dose of albumin-I^{131} were discontinued. In subject T. M. the total exchangeable albumin was calculated from the space of distribution of albumin-I^{131} 7 days following the administration of the second dose. In both instances the mean daily albumin degradation during the last 5 days of the treatment period was assumed to continue into these 4 and 7-day periods. Although the rate of albumin degradation may have been slightly higher than the mean of the previous 5 days, this would not have introduced a significant error in the values for total albumin degraded during thyroid hormone administration.

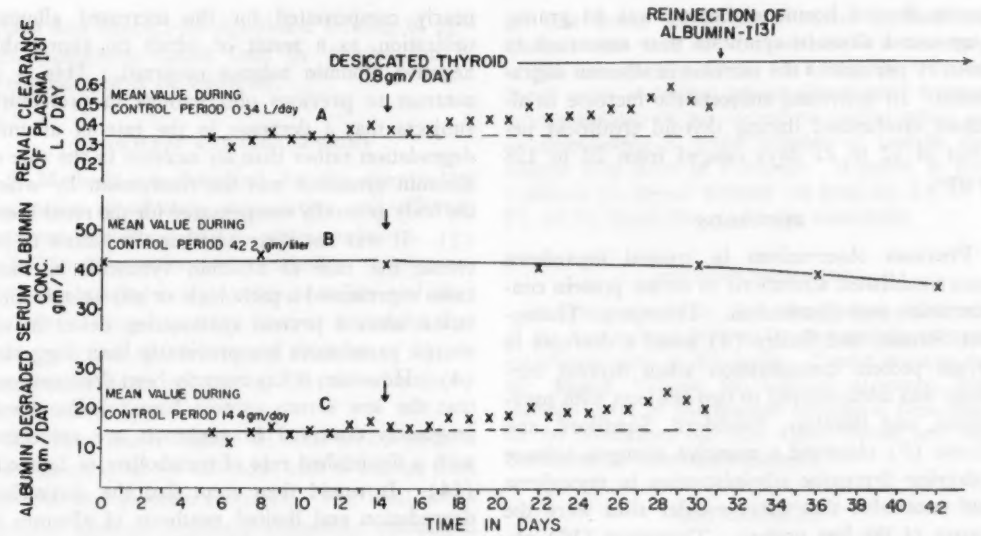

FIG. 2. PATIENT F. M. DAILY URINARY CLEARANCE ("METABOLIC CLEARANCE") OF PLASMA I^{131} (CURVE A), SERUM ALBUMIN CONCENTRATION (CURVE B), AND QUANTITY OF ALBUMIN DEGRADED DAILY (CURVE C)

Following thyroid hormone therapy, there was an appreciable increment in the quantity of albumin degraded even though the concentration of serum albumin decreased.

sized during thyroid administration was obtained from the difference between the total amount degraded and the change in total exchangeable albumin during this period. During the control period the amount synthesized was taken to equal the amount degraded.

RESULTS

Clinical observations and laboratory data which are not related to albumin metabolism are given in the Appendix. It is only necessary to note here that all subjects developed clinical evidence of hypermetabolism akin to that observed in hyperthyroidism within about 2 weeks following initiation of thyroid therapy.

Data pertaining to albumin metabolism are summarized in Table I. During thyroid administration all subjects showed a fall in total serum protein concentration, the mean value decreasing from 7.03 ± 0.20 grams per 100 ml. to 6.26 ± 0.37 grams per 100 ml. Serum albumin concentration fell from 4.62 ± 0.31 grams per 100 ml. to 4.19 ± 0.29 grams per 100 ml. Plasma volume increased in all subjects with a mean change of + 10.4 per cent.

An increase in the overall apparent space of distribution accompanied by a proportionately greater fall in serum albumin concentration resulted in a decrease in TEA of 17 gm. During the control period intravascular albumin was 145 gm. and extravascular albumin 205 gm. After thyroid therapy the values were 143 and 189 gm., respectively, indicating that the slight loss of exchangeable albumin was derived exclusively from the extravascular compartment.

During thyroid administration there was an increased metabolic degradation of albumin in all subjects. This was suggested in the increased urinary excretion of I^{131} (Figure 1, Curves B, D). Although the serum albumin concentration fell, the marked increase in the fractional rate of albumin I^{131} degradation more than compensated for this fall, leading to an increase in the quantity of albumin degraded. Determination of the "metabolic clearance" of albumin I^{131} (Figure 2 and Table I) confirmed the increase in the absolute amount of albumin undergoing metabolic degradation. The mean value of this increase was 81 grams with a range of 32 grams to 165 grams (Table I). Since loss of total exchangeable albumin averaged 17 grams during the same period, the average amount of extra albumin synthesized

during thyroid hormone therapy was 64 grams. Augmented albumin synthesis thus amounted to about 79 per cent of the increase in albumin degradation. In individual subjects the increase in albumin synthesized during thyroid treatment periods of 12 to 22 days ranged from 22 to 128 grams.

DISCUSSION

Previous observations in treated myxedema have established alterations in serum protein concentration and distribution. Thompson, Thompson, Silveus, and Dailey (8) noted a decrease in serum protein concentration when thyroid hormone was administered to two subjects with myxedema, and Boothby, Sandiford, Sandiford, and Slosse (9) observed a negative nitrogen balance following thyroxine administration in myxedema and concluded that extravascular sites were the source of the lost protein. Thompson (10) observed a decrease in blood volume in myxedema which returned to normal with replacement therapy, and Gibson and Harris (11) noted an increased blood volume in hyperthyroid subjects. Schwartz (12) and Lewallen, Rall, Berman, and Hamel (13), employing I^{131} labeled albumin, observed a decrease in extravascular albumin in myxedematous subjects treated with desiccated thyroid. The present study is consistent with these observations and indicates further that the reversal of the abnormalities present in myxedema is not simply referable to correction of a metabolic defect due to lack of thyroid hormone but also that similar changes can be induced by excessive amounts of the hormone even in the absence of such a defect. This is in accord with the widely held concept that thyroid hormone does not produce any qualitative changes in metabolism but acts as a regulator for the quantitative control of autonomous functions. However, the present studies do not rule out the possibility that the changes induced in myxedematous patients and in euthyroid subjects are mediated through qualitatively different mechanisms. Since the precise mechanism of action of thyroid substances has not been definitely established, speculation on this point seems unwarranted at present.

Of special interest is the observation that, under the influence of thyroid hormone, albumin production by the liver increased to a level which nearly compensated for the increased albumin utilization, as a result of which no appreciable negative albumin balance occurred. This is in contrast to previous observations in proteinuric subjects that a decrease in the rate of albumin degradation rather than an increase in the rate of albumin synthesis was the mechanism by which the body generally compensated for the renal losses (2). It was not clear whether the failure to increase the rate of albumin synthesis in these cases represented a pathologic or physiologic limitation, since a protein synthesizing defect in nephrotic proteinuria has previously been suggested (4). However, it has recently been demonstrated that the low serum protein bound iodine levels frequently observed in nephrosis are associated with a diminished rate of metabolism of thyroxin (14). It would then seem that the diminished degradation and limited synthesis of albumin in proteinuria are compatible with normal liver function in a hypometabolic state. The maintenance of a low serum albumin concentration in the presence of significant proteinuria actually minimizes protein loss in the urine and consequent depletion of tissue proteins since albumin excretion would be expected to increase with increased albumin concentration, even if there were no rise in the rate of plasma albumin clearance by the kidneys. Thus, a decrease in albumin degradation without stimulation of albumin synthesis appears to be an economical means of conserving body protein in the presence of proteinuria. Because of the direct stimulation of catabolism, a similar mechanism is not possible in thyrotoxicosis. Hence, the body adapts to protein deficit in different ways depending upon the manner in which this deficit is acquired.

The present study indicates that, at least under the influence of excess thyroid hormone, the normal liver is able to elaborate increased amounts of serum albumin. Whether or not the normal liver can increase its output under euthyroid conditions, assuming the demand is created by increased loss or utilization, cannot be answered by these data. Whipple and Madden (15) observed a rapid restitution of serum protein concentration following plasmaphoresis in dogs and attributed this to an increased rate of protein synthesis. However since the rate of protein degradation was not studied, the possibility that replenishment of protein

stores was effected by significant slowing of protein catabolism rather than by acceleration of synthesis cannot be excluded.

SUMMARY AND CONCLUSIONS

1. Methods are described for the quantitative evaluation of albumin degradation and albumin synthesis under non steady state conditions.

2. The distribution and metabolism of albumin-I^{131} were studied in nine subjects before and after the administration of large doses of desiccated thyroid. Clinical and laboratory evidence of hypermetabolism developed during thyroid administration in all subjects.

3. There was a decline in total serum protein concentration in all subjects with a fall in both albumin and globulin fractions. The total intravascular albumin remained essentially unchanged due to a concomitant increase in plasma volume.

4. The fractional rate and absolute amount of albumin degraded daily increased in all subjects. However augmented albumin synthesis resulted in only a small loss of total exchangeable albumin. This loss was sustained almost entirely by extravascular sites.

APPENDIX

Incidental observations

Since there is a paucity of data on experimental hyperthyroidism in human subjects, the following observations are recorded. In all subjects the first symptoms of hyperthyroidism appeared in about two weeks. Tremor was noted in 7 subjects and heat intolerance in 4. Appetite was noticeably increased in 3 subjects but diminished in 1 patient. None of the subjects developed diarrhea, and only 1 subject noticed an increase in bowel movements. Five of the 9 subjects complained of occipital headache present on arising and lasting for several hours, which is not commonly reported in association with hyperthyroidism. This symptom could not be attributed to hypoglycemia since fasting blood sugar values were normal and the headaches were not relieved by food. There were weight losses of 8 to 19 pounds over the 13 to 25-day periods of thyroid administration in all of the 5 subjects in whom weights were recorded. Blood pressure values remained essentially unaltered during thyroid therapy. The resting heart rate increased to 96 beats per minute or more in 8 of 9 subjects. The basal metabolic rate increased from + 22 per cent to + 49 per cent above the control level with a mean rise of + 37 per cent. In three subjects in whom serum protein bound iodine concentrations were obtained, values ranged from 9 micrograms per cent to 14 micrograms per cent during the period of thyroid administration. Control values were not obtained but the normal range in this laboratory is 3.8 to 7.5 micrograms per cent. Total serum cholesterol concentrations were depressed to approximately 60 per cent of the control values, and no abnormalities in serum bilirubin or cephalin flocculation were noted in 3 subjects. However, in these 3 subjects the thymol turbidity fell from 3.2, 3.4, 1.6 to 1.1, 2.0, 0.5 Shank-Hoagland units, respectively.

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ERYTHROCYTE PRESERVATION. VIII. METABOLIC DEGRADATION OF NUCLEOSIDES *IN VITRO* AND *IN VIVO* [1]

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It has been shown previously that the addition of inosine to ACD preservative [2] prolongs the effective period of *in vitro* storage of erythrocytes (1). Inosine is utilized by the red cell after a phosphorolytic cleavage to ribose-l-phosphate and hypoxanthine, mediated by a nucleoside phosphorylase (2, 3). Ribose-l-phosphate enters the "aerobic shunt pathway" of glucose metabolism after conversion to ribose-5-phosphate, and a subsequent effect is a generation of ATP [3] and the resultant maintenance of the energy reserve of the red cell (4). Inasmuch as the red cell lacks the enzyme, xanthine oxidase (5), it is evident that the other cleavage product, hypoxanthine, is not metabolized further but remains in the red cell and the plasma during storage.

The present investigation is concerned with the rate of conversion of inosine to hypoxanthine by the red cells and the ratio of these substances in the plasma of blood stored in ACDI, as well as the *in vivo* metabolism of inosine after infusion. Related studies on the nucleosides, adenosine and guanosine, will be presented also.

METHODS

Human blood was collected in ACD, and the nucleoside, dissolved in 0.9 per cent NaCl, was added with sterile precautions.

Inosine and adenosine were obtained from Schwarz Laboratories; hypoxanthine and guanosine from Nutritional Biochemicals Corporation.

Hemolysis during storage was determined by the measurement of plasma hemoglobin as the pyridine hemochromogen according to the method of Flink and Watson (6).

Paper chromatography (one-dimensional) on Whatman No. 1 filter paper was performed with the following solvent systems: isobutyric acid: concentrated ammonia: water (66:1:33), n-butanol: water (86:14), and water adjusted to pH 10 with NH_4OH, according to the methods described previously (1). After identification of the purine-containing compounds with the use of an ultraviolet light (Mineralite), the materials were eluted from the paper with water and measured spectrophotometrically. These substances were estimated also by quantitative densitometry [4] of the paper chromatograms.

The hypoxanthine content of the plasma and red cells was determined enzymatically, using xanthine oxidase (7), except that the assays were performed on neutralized PCA filtrates of the various fractions.

Acid filtrates of plasma were prepared in the following way: 2 ml. of plasma were added to 2 ml. of cold 0.6 N PCA, mixed well, and centrifuged at 4° C for 5 minutes at 15,200 × g.[5] The precipitate was washed once with 2 ml. of cold 0.3 N PCA, and the centrifugation was repeated. The supernatant fluid and the washing were combined and neutralized with cold 20 per cent KOH, followed by centrifugation at 4° C for 10 minutes at 15,200 × g. The $KClO_4$ precipitate was washed once with 2 ml. of cold distilled water and centrifugation repeated. This second supernatant fluid and the washing were combined for analysis. Acid filtrates of the red cells were prepared in essentially the same way. Approximately 4 ml. of cells were washed twice with an equal volume of cold 0.9 per cent NaCl each time with centrifugation for 20 minutes at 1,700 × g.[6] The washings were discarded, since they contained only negligible amounts of ultraviolet absorbing materials.

A 50 per cent cell suspension was prepared with 0.9 per cent NaCl. Hematocrit determinations were made on this cell suspension. One ml. of cell suspension was added to 3 ml. of cold 0.6 N PCA, and the neutralization of the acid filtrate proceeded as described above.

[1] This work was supported in part by research grants from the U. S. Atomic Energy Commission and the Office of the Surgeon General, Department of the Army.

[2] Acid-citrate-dextrose, National Institutes of Health, Formula B.

[3] The following abbreviations have been used: ATP = adenosine triphosphate; PCA = perchloric acid; ACDI, ACDA, ACDG, = acid-citrate-dextrose, inosine, adenosine or guanosine, respectively.

[4] Photovolt densitometer, Model 301A, phototube B, filter 5265, wave-length 253 mμ.

[5] Twelve thousand rpm; International Centrifuge Model PRI; high speed attachment; radius from bottom of tube equals 9.5 cm.

[6] Three thousand rpm; International Centrifuge Model PRI; rotor No. 269, radius from center of tube equals 17 cm.

Absorption spectra measurements in the region of 230 to 300 mμ were obtained with the use of the Beckman DU spectrophotometer on the PCA filtrates of plasma or red cells. Since both hypoxanthine and inosine were present in the plasma of blood stored in ACDI or ACDA (1, 4, 8), the relative amounts of each substance could not be estimated directly from light absorption measurements alone. However, after the amount of hypoxanthine had been determined by means of xanthine oxidase, it was possible from the light absorption data on the plasma filtrates, to correct the extinction values at 249 mμ (peak light absorption of hypoxanthine, millimolar extinction coefficient = 10.5) for hypoxanthine concentration. The readings were also corrected for the light absorption of appropriate control samples, *i.e.*, untreated with nucleoside. Thus a value was obtained which was due to inosine. Approximately 400-fold dilutions were made of all samples for spectrophotometric measurement, and the light absorption of the control samples during storage was less than 2 per cent of the nucleoside-treated samples.

Uric acid was determined on plasma and urine by the uricase method of Dubbs, Davis, and Adams (9).

RESULTS AND DISCUSSION

Storage of blood in ACDI

The ratio of hypoxanthine to inosine during the storage of blood in ACDI at 4° C was determined in two experiments. In the first investigation, only the ratio in the plasma was estimated, while the second study involved the ratio of the two compounds in both plasma and red cell fractions.

Experiment 1. Human blood was collected in ACD and divided into two 100-ml. aliquots. After 24 hours of storage at 4° C, 20 ml. of 0.9 per cent NaCl were added to one aliquot, and 20 ml. of inosine solution in 0.9 per cent NaCl (1300, μmoles, *i.e.*, 3,000, μmoles per 100 ml. red cells) were added to the other. Analyses were performed subsequently on days 4, 9, 15, 23, 37, and 57.

There is progressive uptake of inosine by the red cell (*cf.*, Table I), so that by 57 days there are only 14 μmoles of inosine remaining in the plasma, while 1,286 μmoles have been taken up by the cells. Of this amount absorbed, 440 μmoles have returned to the plasma as hypoxanthine, leaving 846 μmoles inside the cells as inosine and hypoxanthine. Paper chromatographic experiments revealed that only two ultraviolet absorbing materials, *i.e.*, hypoxanthine and inosine, were present in the plasma fractions throughout storage. Furthermore, quantitative densitometry of the chromatograms confirmed the ratios of hypoxanthine to inosine given in Table I. The enzyme responsible for the phosphorolytic cleavage of the nucleoside, nucleoside phosphorylase, has been shown previously to be in the soluble portion of the red cells (3).

After phosphorolytic cleavage of inosine, the ribose moiety is metabolized further by the red cell during storage (4), while the nitrogenous base, hypoxanthine, remains unchanged. As the time of storage progresses, hypoxanthine diffuses from the red cell into the plasma fraction in increasing amounts.

Experiment 2. Human blood was collected in ACD and divided into two aliquots: 1) 100 ml. blood + 20 ml. saline; 2) 100 ml. blood + 20 ml. inosine solution (1,272 μmoles, *i.e.*, 3,500 μmoles per 100 ml. red cells). Both aliquots were stored at 4° C for 36 days. The data are reported in Table II. After 36 days of storage there were 742 μmoles of hypoxanthine and 361 μmoles of inosine distributed between the plasma and red cell fractions, or a total of 1,103 μmoles of material. This leaves 169 μmoles (13 per cent of the original amount of inosine) unaccounted for. This loss of material is unexplained, at present, although paper chromatography indicated the presence of an unknown compound capable of absorbing ultraviolet light in the nucleoside-treated sample which was not present in the control erythrocytes.

TABLE I

Plasma hypoxanthine:inosine ratio of blood stored in ACDI

Days' storage	Plasma fraction*,† Inosine (μmoles)	Hypoxanthine (μmoles)	Hypoxanthine:Inosine ratio	Inosine "absorbed" (μmoles)	% Inosine "absorbed"
1	1,300‡				
4	928	143	0.15	372	29
9	709	241	0.34	591	46
15	538	296	0.55	762	59
23	277	298	1.08	1,023	79
37	150	376	2.51	1,150	89
57	14	440	31.40	1,286	99

* The plasma fraction represents the plasma, ACD, and the saline diluent for inosine added to the 100-ml. aliquot of blood (20-ml. inosine solution).

† All values have been corrected for the values of the control sample (100 ml. aliquot of blood stored in ACD + 20 ml. saline).

‡ Thirteen hundred μmoles inosine added after 24 hours' storage.

TABLE II

The relationship of hypoxanthine to inosine in plasma and red cells of blood stored in ACDI

Human blood was stored for 36 days in ACD (100 ml. blood + 20 ml. saline) and in ACDI (100 ml. blood + 20 ml. inosine = 1272 μmoles).

	36 days stored (ACDI)		
	μmoles Is*	μmoles Hx*	Hx:Is ratio
Plasma fraction	330	529	1.6
RBC fraction	31	213	6.9
Total†	361	742	

* Hx = Hypoxanthine; Is = Inosine.
† Total recovery of 1,103 μmoles (361 + 742) is 87% of the original amount. All values are corrected for those of the control (ACD).

Calculations of the concentration of hypoxanthine in either plasma or red cells revealed that at 36 days of storage there was an equilibrium established (approximately 6 μmoles hypoxanthine per ml.).

Storage of blood in ACDA and ACDG

Similar experiments were carried out on blood stored in ACD with the addition of either 1,200 μmoles of adenosine or 1,300 μmoles of guanosine under conditions identical to those described in Experiment 1.

In the study of blood stored in ACDA it was apparent from paper chromatographic analysis that, after 4 days of storage, there was no adenosine in the plasma fraction, and that inosine and hypoxanthine were the only ultraviolet absorbing substances present. In the plasma filtrates throughout storage (*i.e.*, from 4 to 57 days) maximum light absorption occurred between 247 and 249 mμ. By direct measurement, the amounts and ratios of hypoxanthine and inosine were similar to those described for blood stored in ACDI, *e.g.*, on days 4, 9, 15, 23, 37, and 57, the ratios of hypoxanthine to inosine in the plasma were 0.09, 0.23, 0.76, 1.60, 2.20, and 35.3, respectively. Thus, it appears as if storage of blood with adenosine is quite similar to storage with inosine except that the enzymatic conversion of adenosine to inosine *via* the adenosine deaminase (10) results in the liberation of ammonia which accumulates in the blood during storage (1). This conversion of adenosine to inosine is complete after one-hour incubation of stored cells with adenosine (1). The above evidence is confirmatory to previous work from this laboratory (4, 11) and in accordance with the observations of Rubinstein, Kashket, and Denstedt (8).

TABLE III

Hemolysis during storage of blood in ACDI and ACDA

Days' storage	Mg. hemoglobin per 100 ml. plasma fraction		
	Control*	ACDI*	ACDA*
4	29	14	15
9	37	24	35
15	59	34	42
23	121	56	61
37	292	102	107
57	1,296	362	329

* Control (ACD) = 100 ml. blood + 20 ml. saline, ACDI = 100 ml. blood + 20 ml. inosine (1,300 μmoles), ACDA = 100 ml. blood + 20 ml. adenosine (1,200 μmoles).

The degree of hemolysis in blood stored in ACDI and ACDA is compared in Table III. The similarity is apparent, inasmuch as the presence of either nucleoside suppresses hemolysis during storage, although all values are relatively high due to the fact that at each time of sampling, the blood was agitated by thorough mixing.

Analyses of plasma removed from blood stored in ACDG show that guanine is the purine base liberated from guanosine as a result of phosphorolytic cleavage. Guanine is not degraded further during *in vitro* storage. Studies employing paper chromatography reveal that the increasing concentration of plasma guanine during storage is of the same order of magnitude as the amount of plasma hypoxanthine found in blood stored in ACDI. Neither adenosine nor inosine were detected in blood stored in ACDG.

The fate of hypoxanthine and guanine after infusion of their ribosides

It was of interest to investigate the metabolism of purine moieties of inosine and guanosine after intravenous administration of these purine nucleosides into human recipients.

Two experiments were performed in which normal subjects received about 7,000 μmoles of inosine intravenously in a 500-ml. saline infusion. This amount of inosine is equivalent to that required for the preservation of one unit of blood.

TABLE IV

Serum uric acid after inosine infusion

Experiment 1. Normal male subject (99 Kg.) received intravenously 6,960 μmoles inosine over a period of 1.5 hr.

Experiment 2. Normal male subject (103 Kg.) received intravenously 7,470 μmoles inosine over a period of 1.25 hr.

Time after end of infusion	Mg. uric acid per 100 ml. serum
Expt. 1	
0	6.1
5 min.	8.8
45 min.	9.8
3 hr.	7.4
5 hr.	8.4
Expt. 2	
0	6.1
8 min.	11.5
1 hr.	10.0
3 hr.	8.9
5 hr.	8.7
8 hr.	8.5
27 hr.	6.1

Levels of uric acid in serum and urine were determined at various time intervals after the infusion. A transient rise in serum uric acid was produced soon after the infusion, but the value returned to normal in about 24 hours (Table IV).

Uric acid excretion was measured over a period of about 2 days after the infusion and was compared to the normal excretion levels of the subjects. Approximately 32 per cent of the dose of inosine was excreted as uric acid during a period of 24 hours after the infusion (Table V). Subsequent sampling indicated that an additional 10 per cent of the dose was excreted over the next 12 hours at which time the uric acid levels had returned to normal.

Although these experiments are preliminary in nature, it is probable that the series of events which occur *in vivo* after the infusion of inosine are those shown in equation (*1*).

TABLE V

Uric acid excretion after inosine infusion

Experimental conditions as in Table IV

	μmoles uric acid excretion above normal in 24-hour post infusion period*	% dose of inosine excreted as uric acid in 24 hr.
Expt. 1	2,427	34.9
Expt. 2	2,344	31.4

* Normal urinary uric acid was established in the subjects as 3,993 μmoles per 24 hours.

Guanosine (7,060 μmoles) was administered similarly to a patient with leucopenic leukemia, and the data recorded in Table VI indicate that the metabolic end product of the purine base in this case was the same, *i.e.*, uric acid. It is probable that the phosphorolysis and oxidation proceed according to equation (*2*).

Previous studies on the subcutaneous injection of guanosine have demonstrated increments of uric acid excretion in the urine (12). While neither these previous results nor the data presented in this communication permit any conclusions as to the urinary yield of converted nucleoside, they would appear to be within the range observed after the intravenous administration of uric acid alone, *i.e.*, about 60 per cent (13).

TABLE VI

Serum uric acid after guanosine infusion

Patient (58 Kg.) with leucopenic leukemia received intravenously 7,060 μmoles guanosine over a period of one hour.

Time after end of infusion	Mg. uric acid per 100 ml. serum
0	4.4
10 min.	10.9
2 hr.	11.9
3 hr.	12.0
5 hr.	13.3
12 hr.	10.8
20 hr.	6.5

$$\text{inosine} \xrightarrow[P_i]{\text{nucleoside phosphorylase}} \text{hypoxanthine} + \text{ribose-1-phosphate} \xrightarrow[(O_2)]{\text{xanthine oxidase}} \text{xanthine} \xrightarrow[(O_2)]{\text{xanthine oxidase}} \text{uric acid} \qquad (1)$$

$$\text{guanosine} \xrightarrow[P_i]{\text{nucleoside phorphorylase}} \text{guanine} + \text{ribose-1-phosphate} \xrightarrow[H_2O]{\text{guanase}} \text{xanthine} \xrightarrow[(O_2)]{\text{xanthine oxidase}} \text{uric acid} \qquad (2)$$

SUMMARY

After being taken up by erythrocytes, inosine undergoes enzymatic phosphorolysis to yield ribose-l-phosphate and hypoxanthine. The hypoxanthine diffuses outward into the plasma during storage until an equilibrium is reached with that inside the cells.

Adenosine is converted rapidly to inosine during storage and is utilized through the same metabolic pathway, with the resultant increasing concentration of hypoxanthine in the plasma. Guanosine is utilized similarly with the exception that guanine is the purine base which accumulates in the plasma.

The intravenous administration of inosine indicates that the hypoxanthine is oxidized further *in vivo* to uric acid. Likewise, guanosine infusion results in increased concentrations of uric acid in the blood serum.

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EXPERIMENTAL STUDIES OF THE MECHANISMS PRODUCING HYPOCALCEMIA IN HYPERNATREMIC STATES [1]

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That hypocalcemia may occur accompanying or following dehydration in infants has been stressed by Rapoport (1, 2). Several possible mechanisms have been suggested to explain this phenomenon. Rapoport considered the hypocalcemia part of the "post-acidotic syndrome" and thus a part of a readjustment phase following treatment. Hyperphosphatemia secondary to renal impairment could contribute to hypocalcemia as could rapid dilution of the extracellular fluid during therapy. A study of infants with hypernatremic dehydration revealed that hypocalcemia often appeared in this condition prior to therapy and that hyperphosphatemia was not a necessary concomitant (3). Further examination of the data suggested that the serum calcium concentration showed a roughly inverse correlation with the serum sodium concentration. Thus hypernatremia *per se* might be a major causative factor of this hypocalcemia. This report deals with an experimental approach to test the latter hypothesis.

METHODS

The experimental plan was to thirst young rats for a period and then inject sodium salts intraperitoneally; subsequently the animals were to be bled and analyses performed on serum and carcass for electrolytes.

The animals used in the experiments were male albino rats of the Wistar strain weighing between 100 and 200 grams and fed a standard diet. The composition of the diet was: casein, 250 gm., corn starch, 510 gm., vegetable oil, 149.5 gm., Brewer's yeast, 50 gm., U.S.P. salt mixture No. 14, 40 gm., and percomorph liver oil, 0.5 gm. The low potassium diet used in some of the experiments was obtained from Nutritional Biochemicals Corporation.

The intraperitoneal solutions injected (100 ml. per Kg.) contained the following concentrations of ions in mEq. per L. (The designations used below will be continued in the text):

		Na	Cl	HCO_3	K
Isotonic	Na	150	120	30	
Hypertonic	Na	300	240	60	
Hypertonic	Na + K	300	275	75	50

The rats were placed in metabolism cages for urine collection. Except where specified otherwise, the rats were thirsted 24 hours prior to injection and thereafter. A weighed amount of food was left available but it became apparent that the thirsting animals would not eat whether or not they received injections. The animals were sacrificed 24 to 48 hours following injection after anesthetizing with intraperitoneal sodium pentobarbital (50 mgm. per Kg.) and obtaining blood from the abdominal aorta.

Serum was immediately separated from the red cells after clotting and chemical analyses subsequently performed. The chemical methods are those previously reported from this laboratory (3, 4). Sera and urines were analyzed for Na, Ca, Cl, K, Urea N, and osmolarity. The latter determination was made using the Fiske osmometer. In addition P, protein, CO_2 content, and water determinations were performed on the serum. Calcium determinations throughout this study were performed by the method of Harrison and Harrison (5). Carcass analyses were performed by drying the skinned carcass to constant weight and then grinding the dried skin and carcass.

The resultant material was thoroughly mixed and duplicate aliquots were analyzed following ether extraction of the fat. Sodium and potassium were determined by flame photometry after dry ashing. Calculations of the extracellular concentrations of ions were made by correcting for serum water content and by multiplying cation and dividing anion concentrations by the Donnan factor of 0.96. The carcass content of Na, K, and Cl are expressed as mEq. per 100 gm. of fat free solids. Tissue nitrogen is expressed as gm. per 100 gm. of fat free solids. The chloride space (used as an approximation of extracellular fluid) is calculated from the total chloride content of the carcass and the extracellular chloride concentration.

In these experiments the intestinal tract and its contents were included in the carcass analyses in all groups. To assess the possible role of the intestinal content five control animals were analyzed after removal of intestines and compared to five animals simultaneously analyzed

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TABLE I

Results of analyses of sera and carcasses following intraperitoneal injection of hypertonic Na solution into thirsting animals

Serum Ca (*mg./100 ml.*)	ECF Na (*mEq./L.*)	Chloride space (*ml./100 gm. f.f.s.*)	Carcass Na (*mEq./100 gm. f.f.s.*)	Carcass K	Carcass K/N (*mEq./gm.*)	Carcass Na/K (*mEq./mEq.*)
7.4	166	119	30.2	25.3	1.82	1.20
7.5	170	125	29.4	28.4	1.72	1.04
8.8	159	126	25.4	23.0	1.81	1.10
8.6	160	126	26.3	21.8	1.93	1.21
7.8	189	128	27.8	20.4	1.47	1.36
8.3	171	96.3	24.7	24.6	1.57	1.01
7.0	174	101	24.7	23.8	1.61	1.04
7.9	180	100	25.4	24.0	1.64	1.06
7.9	161	119	24.4	23.2	1.91	1.05
8.4	155	132	25.9	23.1	1.65	1.12
8.5	176	121	27.8	22.9	1.62	1.21
Average of 16 thirsting control animals						
10.3 ±0.21	147 ±3.1	116 ±3.0	21.4 ±0.22	26.8 ±0.26	2.22 ±0.05	0.81 ±0.05*

* Standard error of the mean.

in the usual fashion. No significant differences were found for Na, K, Cl, or N content between the two groups. It should also be pointed out that the thirsting rats in the experiments cited had very little intestinal content when sacrificed.

RESULTS

Behavior changes in the thirsting rats were observed though they were not striking. The animals became somewhat withdrawn and lethargic but were hyper-irritable to stimuli. One animal of the sodium loaded group had some muscle twitchings which simulated tetany, but neither the hypocalcemia nor the other chemical derangements in this animal were more pronounced than in others studied. Animals receiving the low potassium diet failed to grow and appeared particularly listless and sick after intraperitoneal injection. In general the changes in behavior observed were not consistently different between thirsting controls and thirsting injected animals, hence such changes seemed largely attributable to thirsting alone.

Hypocalcemia could be regularly reproduced by the intraperitoneal injection of hypertonic sodium solution into thirsting rats. The thirsting state was chosen because in preliminary experiments with similar loads where the animals could drink after injection neither sodium excess nor hypocalcemia could be induced. The time of bleeding is of some importance in detecting hypocalcemia induced in this manner. Animals sacrificed 12 hours following injection did not have hypocalcemia nor did a few surviving rats who were examined at more than 72 hours post injection. Hence the animals discussed herein were all examined between 24 and 48 hours post injection. The pertinent data on eleven such animals with carcass' analyses are presented in Table I and contrasted to the average values obtained from 16 uninjected, thirsting control animals. Though all of the injected animals are hypocalcemic the degree of hypocalcemia does not correlate with the degree of hypernatremia or of body sodium increase. Table II contrasts animals injected with the hypertonic Na solution with three types of controls: uninjected receiving water ad libitum, uninjected and thirsting, and thirsting animals injected with an equivalent volume of isotonic Na solution. The mean serum calcium values in the three control groups are in the normal range. There is no overlapping of calcium levels between

TABLE II

Effect of injection of hypertonic Na solutions (100 ml. Kg.) on serum Ca

Number of animals	Solution	Thirst hours	Mean serum Ca (*mg./100 ml.*)
20	None	None	10.2 ± 0.14*
12	None	48 to 72	10.3 ± 0.22
6	Isotonic Na (150 mEq./L.)	48 to 72	10.2 ± 0.23
27	Hypertonic Na (300 mEq./L.)	48 to 72	7.5 ± 0.38

* Standard error of the mean.

the hypertonic Na group and the other three and all of the calcium values are less than 9 mg. per 100 ml. in the group of experimental animals injected with hypertonic sodium solution. Total serum protein concentrations showed no differences among the various groups; the overall range was from 5.74 to 7.78 gm. per 100 ml. with an average of 6.49 gm. per 100 ml. These values did not differ significantly from unthirsted rats.

Thirsted but uninjected animals lost weight in the order of magnitude of 10 per cent of their initial weight and had very scanty (usually less than 1.5 ml.) urine output. The animals injected with hypertonic solutions showed slight but variable weight loss at 24 hours (1.0 to 6.0 per cent of initial weight) and put out urine volumes from 3.7 to 9.0 ml. in the first 24 hrs. When the experiments were carried to 48 hours the weight losses in the injected group approached those of the thirsted controls though the second 24-hour urine output diminished sharply in amount. The serum urea N concentration rose in all thirsted animals to about 40 mg. per 100 ml. and this concentration was roughly proportional to the length of the thirst. No intergroup differences were noted. Osmolarity determinations carried out on the sera paralleled the sodium concentration in these experiments and appear to add nothing to the data tabulated herein. Urine osmolarity determinations revealed the remarkable ability of the rat kidney to excrete a concentrated urine up to values to 3000 mOsm. per L. Among the animals injected with hypertonic solutions no inter-group differences in osmolarity were noted including the potassium deficient animals.

The extracellular sodium concentration in the experimental group was sometimes only slightly increased above control values. The carcass analyses however make it apparent that total body Na is consistently increased in these animals whereas in some control animals which have slight hypernatremia but without increased Na content, no hypocalcemia occurred. The mean carcass sodium in the experimental animals is 26.6 mEq. per 100 gm. f.f.s. in contrast to a mean of 21.4 mEq. per 100 gm. f.f.s. in the controls. The chloride space calculations showed no significant differences between hypocalcemic animals and controls. A third observation from the carcass data indicated a considerable potassium deficit in the sodium loaded hypocalcemic animals. The first two lines of Table III summarize these data. Control and experimental values for K/N ratios are given to indicate the extent of the potassium deficits. The data suggest that the relative quantities of total body sodium and potassium might be important in determining the observed hypocalcemia. The Na/K ratio which expresses this relationship increased from 0.81 in the controls to 1.13 in the experimental animals. This rise is the result of combined increase of sodium and decrease of potassium.

To test whether potassium deficiency alone might account for the hypocalcemia, rats were made potassium deficient by feeding a virtually potassium free diet for 21 days. This diet which

TABLE III

Comparison of mean serum and carcass analyses on controls, hypertonic Na loads, Na + K loads, K deficiency, and K deficiency plus hypertonic Na loads

Animals (No.)	Serum Ca (*mg./100 ml.*)	ECF Na (*mEq./L.*)	Chloride space (*ml./100 gm. f.f.s.*)	K/N (*mEq./gm.*)	Na/K (*mEq./mEq.*)
Controls (16)	10.3 ± 0.21	147 ± 1.0	116 ± 3	2.22 ± 0.05	0.81 ± 0.05*
Na load— 300 mEq./L. 100 ml./Kg. (11)	8.0 ± 0.17	169 ± 2.5	117 ± 3.8	1.69 ± 0.04	1.13 ± 0.03
Na + K loaded (7)	10.0 ± 0.25	160 ± 1.1	120 ± 3.4	2.38 ± 0.04	0.86 ± 0.02
K deficient (6)	10.0 ± 0.06	147 ± 1.4	120 ± 3.1	1.75 ± 0.04	0.86 ± 0.03
K deficient plus Na load (8)	7.6 ± 0.12	172 ± 1.9	116 ± 3.3	1.43 ± 0.05	1.43 ± 0.05

*S.E.M.

was also low in magnesium content contained 170 mEq. of sodium per Kg. The animals were thirsted for 24 hours and half the group were injected with the hypertonic Na solution. The last two sections of Table III compare these two groups of animals. The uninjected potassium deficient animals were not hypocalcemic, though the chloride spaces were on the average slightly greater than in the injected animals. The ratios of K/N showed the expected decrease. The Na/K ratios were only slightly increased over control animals fed the standard diet (Tables I and III) which may be due to the low magnesium content and the relatively low sodium content of the potassium deficient diet. Cotlove, Holliday, Schwartz and Wallace (6) found that when rats were fed a diet deficient in magnesium intracellular potassium deficit was not accompanied by an increased intracellular sodium such as occurs on a low potassium, high sodium diet. When the intraperitoneal hypertonic Na solutions were given to the potassium deficient animals hypernatremia and hypocalcemia occurred with concentrations of calcium uniformly less than 9 mg. per 100 ml.; there was a further striking reduction in K/N ratio; and finally the Na/K ratio showed the expected marked increase. The experiment indicated that potassium deficiency without sodium excess was not responsible for the observed hypocalcemia.

To test whether sodium excess without potassium deficiency would result in hypocalcemia, another experiment was done in which potassium was added to the sodium loading solution. Seven rats receiving the stock diet were thirsted and injected with the hypertonic Na + K solution. The second and third lines of Table III compare the results in these animals with those in which the hypertonic Na solution was given. The animals given added potassium were hypernatremic but not hypocalcemic; there were no significant differences in the chloride spaces between the two groups; the animals receiving Na + K had normal or increased K/N ratios; and the Na/K ratio is nearly that of the controls despite an absolute increase in carcass sodium. The average value for this sodium was 28.5 mEq. per 100 gm. fat free solids, a clear cut increase over the control value of 21.4 in Table I.

No pH determinations were done in these experiments; however, CO_2 content determinations showed no differences between thirsted controls and the hypertonic Na loaded animals. Values ranged from 16 to 20 mEq. per L. except in animals which had received the potassium free diet. In the latter animals CO_2 levels ranged from 25.8 to 31.4 mEq. per L. again with no differences between the hypertonic Na group and the "controls."

The influence of the serum phosphate concentration may be evaluated by the figure in which the concentration of serum calcium is plotted against the serum phosphorus level for these groups of animals: hypertonic Na loaded, hypertonic Na + K loaded, and thirsting uninjected controls. As previously indicated the animals of the hypertonic sodium group show serum calcium concentrations which are all below 9 mg. per 100 ml. while the others are all greater than this level. On the other hand the serum phosphorus concentrations show considerable overlapping indicating it is not the causative factor for the differences noted in calcium.

Urine examination showed no quantitative differences in calcium excretion between hypocalcemic animals and control animals in those experiments where the experimental design resulted in similar urinary volume for the periods studied. Stools were scanty in all groups. Fecal calcium analyses in a few control and hypocalcemic rats revealed no differences.

DISCUSSION

This series of experiments suggests that an increase in body sodium in the rat will produce a temporary hypocalcemia, provided there is a concomitant body potassium deficiency; neither an excess of sodium nor a deficiency of potassium occurring independently produced this effect. In these experiments the hypocalcemic effect was seen only when the Na/K ratio of the total body was 1.0 or greater. The predisposing effect of potassium deficiency in the development of hypocalcemia in rats following sodium loading has a possible counterpart in infants with hypernatremic dehydration. Rapoport, Dodd, Clark, and Syllm (1) described infants in the recovery phase of diarrheal disease who were hypocalcemic and hypokalemic. A number of the infants with hyper-

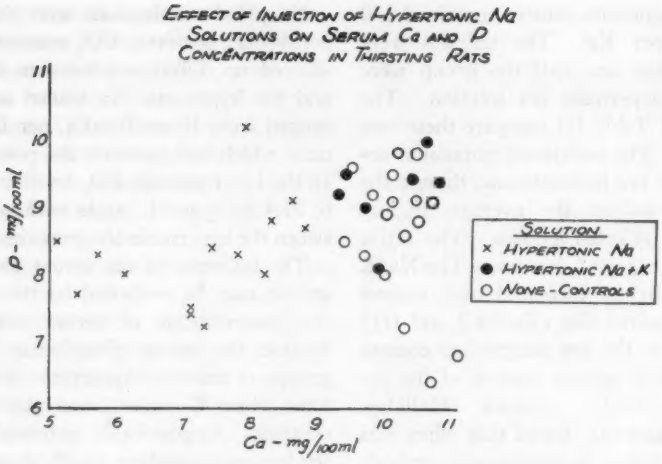
Effect of Injection of Hypertonic Na Solutions on Serum Ca and P Concentrations in Thirsting Rats
P - mg/100ml
Ca - mg/100ml
Solution
Hypertonic Na
Hypertonic Na+K
None - Controls

FIGURE 1

natremic dehydration studied by Weil and Wallace (7) as well as some of those studied in this clinic (3) had hypokalemia even during periods of marked urea nitrogen retention.

The infant with hypernatremic dehydration does not necessarily have hypocalcemia (3). While most such infants probably often have a reduced body sodium content it is possible that some may actually have sodium contents in excess of normal because of previous excessive dietary or therapeutic intake. At present it would be merely speculative to implicate an absolute sodium excess in such infants, or to say that these are the ones who become hypocalcemic. Some of the patients have become hypocalcemic after the administration of large amounts of sodium salts. Depletion of potassium which was found to be a necessary condition in the experimental animal for the occurrence of hypocalcemia seems a probable part of the usual picture of hypernatremic dehydration in the infant (1, 3, 7). The mechanism whereby the potassium acts is not clear.

The concentration of calcium in the extracellular fluid is determined by a number of influences. In this study there appears to be no concern with the portion of calcium bound by serum protein since no differences in protein occurred among the various groups of animals. Changes in intestinal absorption seem unlikely to be significant since little or no food was ingested by the rats once the thirsting period began. Excretion of calcium in the urine showed no differences between the hypocalcemic animals and the others. Thus rapid calcium excretion also seems unlikely to be a factor in this study.

The data suggest that the effect demonstrated by this study results from an alteration in the equilibrium point of the balance between extracellular calcium and the skeletal calcium. The steady state between dissolved calcium and calcium of bone salt probably involves cellular activity of skeletal tissue as well as a physico-chemical equilibrium between solution and solid phases. Parathyroid hormone and vitamin D probably influence the cellular activity involved, but the present data are not adequate to show whether the effect might be mediated through a disturbance in those systems. Sodium is considered to accumulate on the surfaces of bone crystals and this sodium is known to act as a sort of flexible sodium reservoir under conditions of physiologic disturbances of electrolyte equilibrium (8, 9). Sudden increases in sodium content of bone surface may well play an interfering role in the maintenance of calcium homeostasis. No data bearing on this point are contained in the present study. Moreover the "protective" role of the potassium would be difficult to explain since the sodium content of the Na + K loaded animals is as high as those loaded with hypertonic Na. Additional experiments are needed to further elucidate the mechanisms involved.

SUMMARY AND CONCLUSIONS

Thirsting rats injected with hypertonic sodium solution become hypocalcemic concomitant with a rise in total body sodium and a fall in body potassium. Neither extracellular dilution nor phosphate retention were important factors in producing this effect. Sodium excess alone or potassium deficiency alone does not result in the hypocalcemia. The total body sodium was equal to or greater than the total body potassium (measured in milliequivalents) in the animals which developed hypocalcemia. It is suggested that the hypocalcemia results from an alteration in equilibrium between extracellular and skeletal calcium.

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STUDIES OF RESPIRATORY PHYSIOLOGY IN THE NEWBORN INFANT. III. MEASUREMENTS OF MECHANICS OF RESPIRATION [1]

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Considerable attention has recently been focused on the mechanical factors in respiration of normal adults and of those with respiratory abnormalities. This report presents observations on the mechanics of respiration in 23 normal newborn infants and 2 infants critically ill with neonatal respiratory distress. The data are derived from simultaneous measurements of tidal volume and intraesophageal pressure changes.

MATERIAL AND METHODS

The infants, all of whom were born at the Boston Lying-in Hospital, weighed from 2.4 to 3.8 Kg. at birth and were from 1 hour to 7 days old at the time of study. History, physical examination and, in most cases, chest x-rays were used to determine presence or absence of respiratory distress. On the basis of observation and previously established criteria (1), 18 of the normal infants were considered to have been studied during periods of quiet, resting respiration. In all, 47 studies were made on 28 [5] newborn infants ranging from the 18 infants breathing quietly to those who were sick or disturbed by the procedure. The respiratory rates varied from 24 to 136 per minute.

The two infants studied when critically ill and during recovery were diagnosed as having the neonatal respiratory distress syndrome. This syndrome, which is also called the hyaline membrane syndrome or resorption atelectasis, is characterized by a history of premature birth, cesarean section, fetal distress, or maternal diabetes and the clinical picture of increasing respiratory difficulty, cyanosis, and frequently typical x-ray findings (2).

The infants were placed in a 65-liter body plethysmograph (Figure 1) with their faces emerging through a pneumatic cuff. Pressure changes within the plethysmograph for an average respiration were approximately 0.3 cm. H_2O and were measured by an electrical manometer (3). With a calibrated syringe and pump, breathing was simulated and the pressure changes were calibrated in terms of volume. Although this calibration was performed after the infant was removed, the resulting error was less than 5 per cent and was therefore not taken into consideration in the calculation. Intraesophageal pressure changes as indices of intrapleural pressure changes were measured with a small water-filled polyethylene catheter (internal diameter 1.0 mm.) passed 10 to 11 cm. through the nose or mouth into the esophagus and connected to a second manometer [6] calibrated in cm. H_2O. When inserted to this distance, the open catheter tip was shown by x-ray of two infants to be at the junction of the middle and upper thirds of the esophagus. Volume and pressure were recorded simultaneously on a direct-writing oscillograph.[6]

Pulmonary compliance was expressed as the ratio of tidal volume to the change in intraesophageal pressure measured between points of no flow, *i.e.*, at the extremes of tidal volume (Figure 2). Respiratory resistance was measured as the ratio of the total pressure change to the corresponding total flow change between points of equal volume approximately midway in inspiration and expiration (Figure 3). This calculation of resistance has provided a satisfactory approximation of the average flow-resistance of the lungs and air passages during the respiratory cycle in adults (4). The average compliances of the individual infants were calculated from 10 to 20 representative respirations and average resistances from 5 to 10 respirations (Table I).

From the simultaneous recordings of pressure and volume, pressure-volume loops for the respiratory cycle were plotted and from 3 to 6 representative breaths a graphic solution of average work done on the lungs per breath was obtained [7] (Figure 4). As indicated in the

[1] One of a series of studies supported by a research grant from the Association for the Aid of Crippled Children, New York City.

[2] Public Health Service Research Fellow of the National Heart Institute.

[3] Traveling Fellow of the R. Samuel McLaughlin Foundation, Canada.

[4] Traveling Fellow of the British Post-Graduate Medical Fellowship.

[5] Although the data are not included in Table I, determinations of work of respiration of one newborn with congenital heart disease and two with borderline respiratory distress are included in Figures 7 and 8 for comparison of the three methods used to calculate pulmonary work.

[6] An electromanometer and the Polyviso made by Sanborn Company, Cambridge, Massachusetts, were used.

[7] As pointed out in the discussion, these measurements do not allow calculation of *total* "work done on the lungs" but do allow an apparently adequate approximation.

a - Pneumatic cuff
b - Intraesophageal catheter
c - Stopcocks
d - Krogh spirometer
e - Tidal and minute volumes
f - Electric timer
g - Kymograph
h - Electrical manometer
i - Calibration - syringe and pump

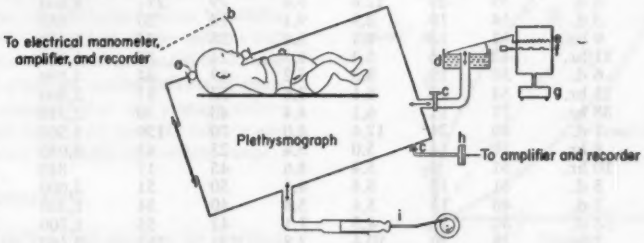

FIG. 1. DIAGRAM OF APPARATUS USED FOR RESPIRATORY STUDIES

With the stopcock to the spirometer closed, the apparatus was used as a pressure plethysmograph. With the stopcock open to the spirometer, minute volume was recorded with a photoelectric integrator (1).

diagram, this method of measuring work allows separation of pulmonary work into elastic and flow-resistive components. For purposes of comparison, pulmonary work for the same respirations was also estimated, using two formulae:

1. A simplified formula was suggested by one of the authors, (M. B. McI.), as a possibly adequate approximation of work done on the lungs during inspiration and expiration.

$$\text{Work (in gm. cm. per min.)} = 0.6\ P\dot{V},$$

where P = total pressure change in cm. H_2O during the respiratory cycle.

$\dot{V}$ = minute volume in ml.

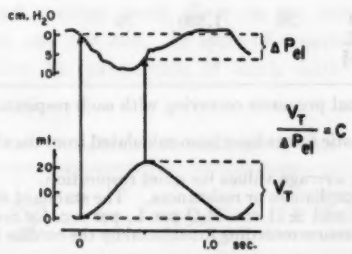

FIG. 2. METHOD OF CALCULATING PULMONARY COMPLIANCE

As shown in this diagrammatic representation of simultaneous pressure and volume recordings, compliance (C) is expressed as the ratio of tidal volume (V_T) to the change in intraesophageal pressure (P_{es}) measured between points of no flow, *i.e.*, at the extremes of tidal volume.

This formula is based on the fact that, if the intraesophageal pressure is represented by a sine wave, purely elastic work would be represented by the formula 0.5 $P\dot{V}$ (the area of a triangle), purely viscous work by the formula 0.79 $P\dot{V}$ (the area of an ellipse), and the fact that approximately 70 per cent of pulmonary work in normal adult respiration is elastic (5).[8]

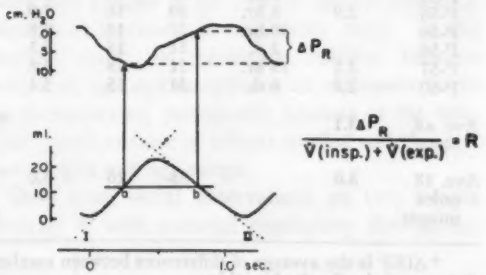

FIG. 3. METHOD OF CALCULATING FLOW RESISTANCE

Respiratory resistance (R) is measured as the ratio of total pressure change (P_R) to the corresponding total flow change ($V_{insp} + V_{exp}$) between points of equal volume (points a and b). Total flow change between points a and b was obtained by measuring the slopes (lines I and II) of the volume curve at these points.

[8] The expression 0.6 $P\dot{V}$ should not be confused with the expression 0.7 Pmax$\dot{V}$ used by McIlroy and Eldridge (6) to obtain an approximation to the work of inspiration. In that case Pmax was the maximum pressure difference during inspiration. In the expression 0.6 $P\dot{V}$ used in this paper, P is the total intrathoracic pressure swing.

TABLE I

Data from studies on the mechanics of respiration in 23 normal newborn infants

No.	Birth wt. Kg.	Age	Resp. rate per min.	Tidal vol. ml.	ΔIEP* cm. H_2O	Compliance mean ml./cm. H_2O	Resistance mean cm. H_2O/L./sec.	Work per breath† gm. cm.	Work per minute† gm. cm.	% Elastic work†	Comments
P-30	3.7	6 d.	39	29	12.6	3.8	79	217	8,460	51	Restless
P-31	3.1	3 d.	44	16	2.9	9.1	13	20	885	70	Quiet‡
P-32	2.8	9 hr.	41	13	4.4	3.4	28	33	1,350	76	Quiet‡
P-33	3.1	11 hr.	42	15	5.4	4.3	24	36	1,490	74	Quiet‡
P-33		6 d.	38	19	8.3	3.2	49	84	3,190	67	Restless
P-34	2.4	15 hr.	54	13	6.1	2.6	53	53	2,860	62	Quiet—not basal
P-34		38 hr.	77	11	6.1	4.4	43	30	2,310	46	Restless
P-34		7 d.	40	24	12.4	4.0	70	139	5,560	52	Restless
P-35	3.8	8 hr.	70	14	5.0	3.4	25	43	3,010	67	Quiet—not basal
P-38	2.4	10 hr.	51	9	5.4	4.6	45	17	840	53	Quiet‡
P-39	3.4	5 d.	51	15	8.4	4.1	50	51	2,600	54	Restless
P-41	3.0	2 d.	40	15	5.4	5.8	40	34	1,360	57	Quiet‡
P-42	3.5	3 d.	31	21	4.5	7.1	42	55	1,700	57	Quiet‡
P-43	3.6	2 hr.	28	20	10.4	3.8	131	113	3,160	47	Quiet
P-43		4 hr.	48	15	4.1	5.1	18	30	1,445	73	Quiet‡
P-44	2.7	12 hr.	38	14	5.5	3.9	45	39	1,480	65	Quiet‡
P-45	2.9	16 hr.	24	25	5.3	9.3	43	60	1,440	56	Quiet‡
P-47	2.5	4 hr.	35	11	3.3	4.0	24	19	675	78	Quiet‡
P-47		22 hr.	136	10	3.2	3.2	25	30	4,080	52	Restless
P-48	2.8	3 hr.	42	16	5.6	3.7	21	44	1,850	79	Quiet—not basal
P-48		22 hr.	37	18	2.9	8.6	7	22	810	85	Quiet‡
P-49	3.0	2 d.	41	18	4.5	7.4	17	31	1,270	71	Restless
P-51	3.3	1 hr.	44	13	7.7	2.2	7	60	2,635	64	Quiet‡
P-51		23 hr.	66	12	5.3	3.7	24	29	1,910	67	Restless
P-53	3.5	8 hr.	57	14	4.0	5.4	13	24	1,370	75	Restless
P-53		2 d.	49	12	5.2	2.5	36	39	1,925	73	Quiet‡
P-53		3 d.	98	28	5.9	6.8	22	126	12,100	46	Very restless
P-53		6 d.	53	15	4.6	4.6	32	40	2,120	61	Restless
P-54	3.1	21 hr.	29	21	3.9	7.9	13	35	1,020	80	Quiet
P-54		2 d.	25	19	5.2	6.9	21	34	850	77	Quiet‡
P-54		6 d.	27	24	5.5	7.1	37	64	1,700	63	Quiet
P-55	3.5	2 d.	64	13	6.7	5.9	75	48	3,070	30	Restless
P-55		3 d.	35	19	5.3	4.9	35	55	1,925	67	Quiet‡
P-56	2.9	6 hr.	30	16	7.0	3.4	19	44	1,310	86	Quiet‡
P-56		30 hr.	37	15	9.8	3.3	80	62	2,300	55	Restless
P-56		3 d.	51	15	5.5	3.1	41	56	2,840	65	Quiet—not basal
P-57	3.1	13 hr.	31	18	5.8	4.8	39	50	1,545	68	Quiet‡
P-60	2.8	6 d.	31	15	5.4	3.9	33	38	1,190	75	Quiet‡
Ave. all obs.	3.1					4.9					
Ave. 18 quiet infants	3.0		38	16	5.0	5.2 (S.E. ±0.4)§	29 (S.E. ±4)§	38	1,380	70	

* ΔIEP is the average of differences between maximal and minimal pressures occurring with each respiratory cycle during periods of quiet breathing.

† Work per breath and per minute and per cent of work against elastic forces have been calculated from the simplified formula of Otis, Fenn, and Rahn (7).

‡ These 18 studies on 18 different infants were used for obtaining average values for quiet respiration.

§ S.E. = standard error of the mean of a series of 10 individual compliances or resistances. The standard deviation of a single determination was ±1.28 ml. per cm. H_2O for compliance and ±11 cm. H_2O per L. per sec. for resistance. These large individual variations are apparently due to the artifact in pressure recording introduced by the cardiac impulse.

2. Work was also calculated by substituting the determined elastic and resistive factors and tidal volume and respiratory rate in the formula of Otis, Fenn, and Rahn (7). In doing this, it was necessary to assume that the second order resistive factors were negligible. Thus the formula actually used was:

Work (in gm. cm. per min.)

$$= \tfrac{1}{2} f K_{el} (V_T)^2 + \tfrac{1}{2} K_r \pi^2 f^2 (V_T)^2,$$

where $K_{el} = \dfrac{1}{\text{compliance}}$ with compliance expressed as ml. per cm. H_2O

V_T = tidal volume in ml.

f = breaths per min.

K_r = resistance in cm. H_2O per ml. per min.

Since in some infants the introduction of the intra-esophageal catheter was followed by an increase in respiratory rate, the minute volume, rate, and tidal volume were obtained in most cases before or after the catheter was in place, utilizing a previously described technique for recording rate and minute volume (1) (see Figure 1). The resting rate and tidal volume were used in the simplified Otis formula for calculating the pulmonary work of the 18 quiet infants. Since these 18 infants had, when breathing quietly, an average respiratory rate of 38 per minute and an average minute volume of 570 ml. compared to 33 and 550 observed in another group of resting infants of comparable size (1), it was assumed that the average calculated pulmonary work per minute of these 18 infants approximated that of newborn infants in this weight range.

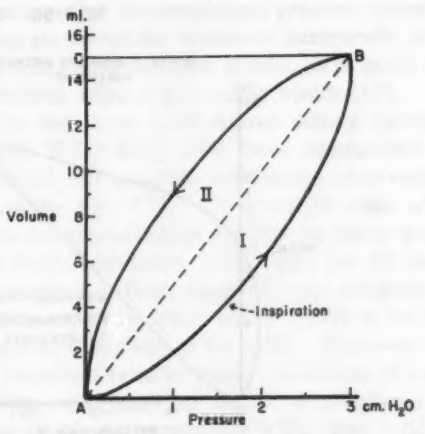

FIG. 4. DIAGRAMMATIC AVERAGE NORMAL PRESSURE-VOLUME RESPIRATORY LOOP

Elastic work is represented by area of triangle ABC. Inspiratory and expiratory flow-resistive work are represented by areas I and II, respectively. Assuming that expiration is passive, total pulmonary work is represented by the sum of the elastic work (triangle ABC) plus the inspiratory flow-resistive work (area I).

RESULTS

The results of the individual studies on normal infants are presented in Table I. The 23 normal infants averaged 3 Kg. in weight. The average tidal volume of the quiet infants was 16 ml. (range 9 to 25). Peak flow rates for individual infants averaged 61 ml. per sec. (range 44 to 111 ml. per sec.) during quiet respiration. The average compliance from 38 studies on the 23 normal infants was 4.9 ml. per cm. H_2O and for the 18 resting infants was essentially the same (5.2 ml. per cm. H_2O). Although there was considerable variability in both compliance and resistance determinations from breath to breath due to the cardiac component of the pressure recording, the standard error of the mean of a series of 10 compliances was only $\pm$ 0.4 ml. per cm. H_2O. The mean resistance was 29 cm. H_2O per L. per sec. (standard error of the mean of a series of 10 = $\pm$ 4 cm. H_2O per L. per sec.) for these 18 infants and the calculated average work done on the lungs was 1380 gm. cm. per min. In spite of occasional discrepancies, the proportion of work done against elastic forces estimated from the pressure-volume diagram and from the Otis formula showed on the average a close correlation, being 71 and 70 per cent, respectively. As was expected, pulmonary work was greater in the restless infants primarily because of increases in respiratory rates. In the present study no significant relation between weight or age and compliance or resistance could be demonstrated, presumably because of the relatively small number of infants studied and the narrow weight and age range.

Data from serial observations on two infants severely ill with neonatal respiratory distress are

TABLE II

Data on the mechanics of respiration in two infants with neonatal respiratory distress

No.	Birth wt. (Kg.)	Age	IEP* (cm. H_2O)	Compliance (ml./cm. H_2O) Mean	Compliance Range	Resistance (cm. H_2O/L./sec.) Mean	Resistance Range	Work per breath† (gm. cm.)	Work per minute†† (gm. cm.)	Per cent elastic work†	Comments
P-40	3.0	2 d.	16.3	1.3	1.3–1.5	25	22–29	144	7,900	89	Severely ill
P-40		6 d.	4.3	2.5	1.8–3.6	13	5–23	25	1,430	80	Recovering
P-50	3.2	5 hr.	20.0	0.7	0.6–0.8	41	0–104‡	107	5,130	71	Severely ill
P-50		2 d.	20.8	1.0	0.8–1.3	13	0–27‡	176	7,400	84	Severely ill
P-50		10 d.	7.7	2.5	2.2–3.0	39	18–65	64	3,160	73	Recovering

* IEP is the average of differences between maximal and minimal pressures occurring with each respiratory cycle.

† Pulmonary work per breath and per minute and per cent elastic work were estimated directly from the graphic pressure-volume loops for these two sick infants.

‡ Resistance calculated only on inspiration because of grunting expiration.

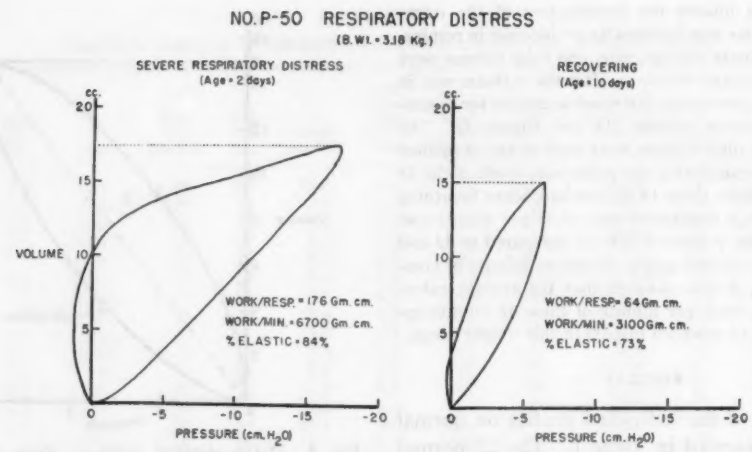

FIG. 5. PRESSURE-VOLUME LOOPS FOR INFANT WITH RESPIRATORY DISTRESS

The marked increase in pulmonary work during respiratory distress is apparent. There is no increase in inspiratory flow resistance although the expiratory flow resistance is increased as a result of the grunting respiration.

shown in Table II. A typical pressure-volume loop for one of the infants during severe distress and a second loop during recovery are shown in Figure 5. These infants have, in contrast to normal newborn infants, a marked decrease in pulmonary compliance to 15 to 20 per cent of normal. Because of this and an increased respiratory rate, the work of respiration is increased by more than 400 per cent.

DISCUSSION

As in the one other report on the mechanics of breathing in newborn infants (8), the present data and calculations have been based on the assumption that intraesophageal pressure changes were reliable indices of intrapleural pressure changes. Although this concept has been based on studies in adults, the technique has a number of limitations in adults as well as newborn infants.

Data of Cherniack, Farhi, Armstrong, and Proctor (9) show that this indirect technique does not provide a reliable measure of absolute intrapleural pressure even in adults. However, their data indicate that pressure changes determined directly and indirectly during quiet breathing were on the average comparable. Secondly, recent work has shown that both intrapleural and intraesophageal pressure changes are influenced by the position of the patient, being more reliable and consistent in the upright position than in the supine (10). This position factor may or may not be applicable to studies in the newborn. In any case it would be difficult to study infants in any other than a supine position.

One further source of error may be related to the use of a water-filled catheter rather than an air-filled balloon. In a comparative study, Mead, McIlroy, Selverstone, and Kriete (11) have shown that the balloon is superior but the differences were least in the upper portion of the esophagus. In

TABLE III

Average data on mechanics of respiration for newborn infants and adults

	Infant	Adult
Weight (*Kg.*)	3.0	70
Respiratory rate (*per min.*)	38	15
Compliance (*ml./cm. H_2O*)	5.2 (2–9)	170 (Ref. 14)
Compliance (*per gm. lung*)	.08	.15
Compliance (*per ml. V_{FRC}*)*	.065	.063
Resistance (*cm. H_2O/L./sec.*)	29 (7–45)	2 (1–3) (Ref. 17)
Pulmonary minute work (*gm. cm.*)	1,380	15,700†
Per cent elastic work	71	67†

* Based on V_{FRC} from references 15 and 16.

† Calculated from simplified formula of Otis, Fenn, and Rahn (7).

the present work, this error was presumably minimized by placing the tip of the catheter in the upper third of the esophagus.

In regard to the pulmonary work calculations, it has been pointed out by Otis (12) that the work represented by the pressure-volume diagram described above is not the total work done on the lungs. To the extent that end-expiratory intrapleural pressure is less than atmospheric, additional elastic work must be done during inspiration. Part of the energy for this additional work is supplied by the elastic recoil of the thorax, and the remainder by the respiratory muscles. A more complete measurement of pulmonary work can be accomplished only if both the absolute intrapleural pressure and the elastic characteristics of the thorax are known.

Obviously, further observations comparing intrapleural and intraesophageal pressure changes in infants and studies of the effect of various factors on these measurements are desirable, but difficult to obtain. Pending these, it seems necessary to assume that intraesophageal pressure measurements are acceptable indices of intrapleural pressure changes in newborn infants, as in small experimental animals (3) and in adults (13).

The data from quiet normal infants (average weight = 3.0 Kg.) have been summarized in Table III and compared with similar observations for adults (14, 17). On a weight basis adult lungs are approximately 18 times as heavy as infant lungs. However, adult lungs are 33 times as compliant. Thus, expressed per kilogram of lung tissue, infant lungs would appear to be less compliant than those of the adult. However, using functional residual volume as a basis of comparison, adult lungs are similar in compliance to those of infants (compliance/FRC ratio = 0.065 in infants and 0.063 in adults).

While comparison of compliance can logically be made on the basis of lung weight or lung volume, adult and infant lung resistances are more difficult to compare. Even the comparative effect on flow resistance of nose-breathing in the infant

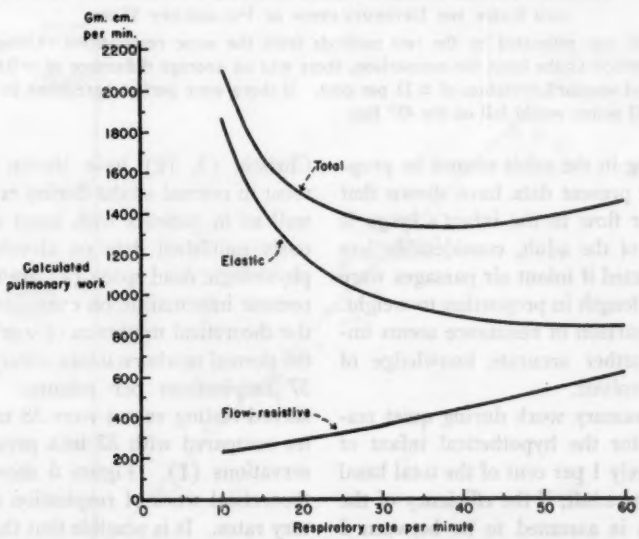

FIG. 6. CALCULATED PULMONARY WORK VERSUS RESPIRATORY RATE

Using the present data on mechanics of respiration in newborn infants and previously determined values for respiratory dead space (5.5 ml.) and alveolar ventilation (385 ml.), the theoretical pulmonary work at constant alveolar ventilation was calculated for various respiratory rates from the simplified formula of Otis, Fenn, and Rahn (7). The value used for compliance was 5.2 ml. per cm. H_2O and for resistance was 29 cm. H_2O per L. per sec. The theoretical minimum work of respiration occurs at a rate of 37 per minute.

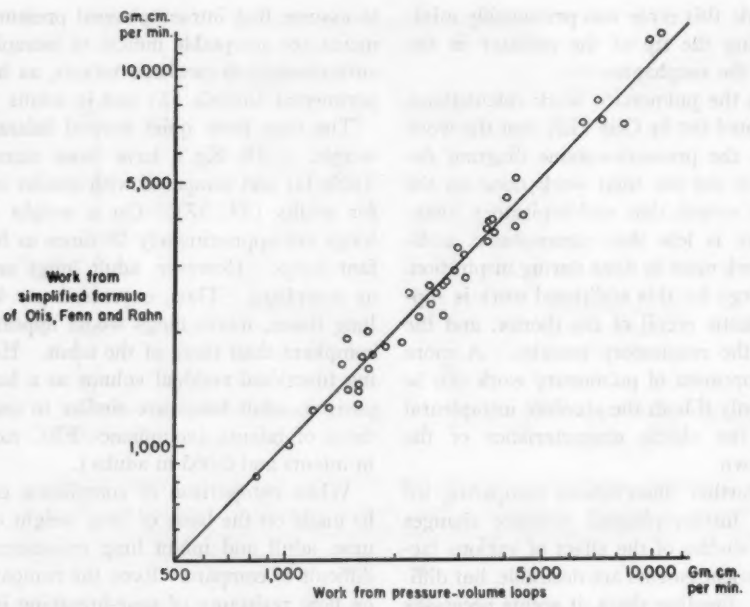

FIG. 7. PRESSURE-VOLUME DIAGRAM VERSUS SIMPLIFIED FORMULA OF OTIS, FENN, AND RAHN FOR DETERMINATION OF PULMONARY WORK

Work was estimated by the two methods from the same respirations. Using the loop method as the basis for comparison, there was an average difference of -0.6 per cent and standard deviation of ± 11 per cent. If there were perfect agreement in each case, all points would fall on the 45° line.

and mouth-breathing in the adult cannot be properly assessed. The present data have shown that the resistance to air flow in the infant's lungs is only 15 times that of the adult, considerably less than would be expected if infant air passages were reduced in size and length in proportion to weight. More detailed comparison of resistance seems impossible without further accurate knowledge of the many factors involved.

The average pulmonary work during quiet respiration estimated for the hypothetical infant of 3 Kg. is approximately 1 per cent of the total basal metabolism, as in the adult, if the efficiency of the respiratory muscles is assumed to be between 5 and 10 per cent for both age groups (7).

It has been shown by Otis, Fenn, and Rahn (7) that the rate and depth of breathing in normal adults are adjusted with the result that alveolar ventilation is accomplished with the minimum expenditure of total respiratory work. When only pulmonary work was estimated by the pressure-volume method, McIlroy, Marshall, and Christie (5, 18) have shown a similar adjustment in normal adults during rest and exercise, as well as in patients with heart disease. If previously published data on alveolar ventilation and physiologic dead space (1) are combined with the present information on compliance and resistance, the theoretical minimum of work of respiration in the normal newborn infant occurs at approximately 37 respirations per minute. The average observed resting values were 38 in the present studies compared with 33 in a previous series of observations (1). Figure 6 shows graphically the theoretical work of respiration at various respiratory rates. It is possible that the wide variation in normal respiratory rates recorded for newborn infants is related to the small change in pulmonary work between respiratory rates of 30 and 50 per minute.

In addition to establishing elastic and viscous resistance constants and approximations of the pulmonary work for newborn infants, the present study allowed comparison of the three meth-

ods of calculating pulmonary work. The results of these comparisons are shown in Figures 7 and 8. It can be seen that the three methods have a close correlation over the wide range of compliance, resistance, respiratory rate, and tidal volumes observed. Using the graphic solution of the pressure-volume diagram as the basis for comparison, the formula of Otis, Fenn, and Rahn gave an average difference of − 0.6 per cent with a standard deviation of ± 11 per cent. This close correlation suggests that second order resistive factors are relatively unimportant in the respiration of newborn infants. On the same basis of comparison, the simplified formula (0.6 PV̄) differed on the average by − 6 per cent and showed a standard deviation of ± 13 per cent. These data show that, at least in infants, pulmonary work can be approximated by application of a simplified Otis formula and even with considerable accuracy on the basis of simple measurements of pressure and volume.

This series of studies on newborn infants was undertaken not only to investigate respiratory physiology, but also to try to delineate the pathophysiology of neonatal respiratory distress. The results of observations on two infants initially studied during severe neonatal respiratory distress (or the hyaline membrane syndrome) and again during recovery have shown that respiratory difficulty of this sort is accompanied by a marked decrease in compliance to 1.3 ml. and to 0.7 ml. per cm. H_2O, a moderate increase in viscous work and a marked increase in the work of respiration. These observations, which have been borne out by non-simultaneous pressure-volume studies in three infants and pressure studies alone in 17 others with respiratory distress (19), would appear to explain the marked sternal retraction observed clinically. As one would predict from the compliance and resistance measurements in these sick infants (if the minimum work concept of Otis, Fenn, and Rahn is applicable), they adjust to their pulmonary insufficiency by increasing their respiratory rate with relatively little change in tidal volume (19). Presumably, another

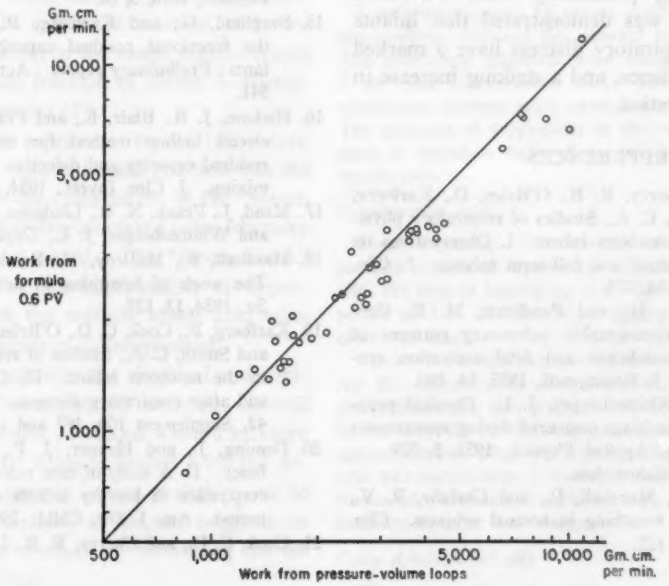

FIG. 8. PRESSURE-VOLUME DIAGRAM VERSUS 0.6 PV̄ FORMULA FOR DETERMINATION OF PULMONARY WORK

Work was estimated by the two methods from the same respirations. Using the loop method as the basis for comparison, there was an average difference of − 6 per cent and standard deviation of ± 13 per cent. If there were perfect agreement in each case, all points would fall on the 45° line.

result of the decreased compliance is the inability of these infants to achieve more than approximately half of normal "crying vital capacity" (20, 21). The demonstration of a marked increase in the work of respiration in neonatal respiratory distress supports the clinical impression that these infants frequently die of exhaustion and indicates that, until this condition can be prevented or specifically treated, therapy should, at least in part, be directly toward support of respiratory efforts.

SUMMARY

In summary, 43 observations on the mechanics of respiration in 23 normal newborn infants and 2 infants with respiratory distress have been reported. The resistance for an average 3-Kg. infant breathing quietly was found to be 29 cm. H_2O per L. per sec. and the average compliance 5.2 ml. per cm. H_2O. The resting pulmonary work for such an infant was approximately 1,400 gm. cm. per minute or 1 per cent of basal metabolism. In addition, it was shown that three methods of calculating pulmonary work correlated well. Finally, it was demonstrated that infants with neonatal respiratory distress have a marked decrease in compliance, and a striking increase in the work of respiration.

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A NEW METHOD FOR MEASURING SIALIC ACID LEVELS IN SERUM AND ITS APPLICATION TO RHEUMATIC FEVER [1]

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Previous reports (1–4) have indicated the clinical usefulness of a color reaction given by serum with the diphenylamine reagent of Dische. It has been shown that the purple color produced in this reaction is probably due to the presence of a deoxyhexose structure in the sialic acid molecule (5). Sialic acid, or a closely related substance, appears to be a common constituent of mucoproteins (glycoproteins) including orosomucoid of serum (6, 7). The color produced in the diphenylamine reaction reflects the concentration of the mucoproteins in the serum being tested. In our experience, and as reported by Anderson and Maclagan (8), the color developed with diphenylamine appears to be a specific reaction of mucoproteins. An elevation in the mucoproteins found in the alpha globulin fraction of serum is characteristic of many diseases (7).

As pointed out in a previous report (4) a constant feature of the diphenylamine reaction was the concomitant rise in the color index of the "serum blank" associated with the increased color intensity of the serum samples tested. The "serum blank" in this instance was the combination of deproteinized serum with the sulfuric acetic acid mixture which did not contain diphenylamine.

This report will consider the chemical and clinical aspects of the "serum blank" reaction. It will be shown that the color produced when a mixture of sulfuric and acetic acids is heated with the deproteinized serum sample is also probably due to sialic acid. The simplicity of the test suggests its usefulness as a new method for measuring the intensity of inflammation.

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METHODS

The apparatus and procedures used in the electrophoretic studies have been previously described (9). Hexose analyses were made with the anthrone reagent according to the method of Dreywood (10) as modified by Morris (11), and Seifter, Dayton, Novic, and Muntwyler (12). A solution containing equal parts of galactose and mannose was used as a standard. Limitations of the anthrone method have been discussed by Sattler and Zerban (13) and by Yemm and Willis (14). Nitrogen and hexosamine determinations were made as described earlier (9). Sialic analysis was performed colorimetrically with the Dische diphenylamine reagent using sialic [3] acid as a standard.

CONDITIONS OF THE REACTION

Interference caused by serum proteins other than the mucoproteins required the removal of these substances. The interfering materials were found to precipitate in 5 per cent trichloracetic acid. Indoles, pyrroles and tryptophan, produce color when heated with the reagent. The presence of tryptophan in the various serum proteins is therefore believed to be the chief source of interference.

Precipitation step

Time of heating. The intensity of the color obtained per unit volume of supernatant solution increased rapidly with the time of heating up to 5 minutes. For a heating period of 5 to 20 minutes no difference in intensity of color was found.

Trichloracetic acid concentration. The effect of changing the concentration of trichloracetic acid during the precipitation step was studied. No difference was found in the quantity of chromogenic material in the supernatant solution when the concentration of trichloracetic acid was varied from 5 to 20 per cent. With a concentration of trichloracetic acid less than 5 per cent a lower intensity of color was obtained.

Color development step

The concentration of sulfuric acid in the reagent was varied from 2.5 per cent to 40 per cent. Maximum color development occurred with a reagent containing 5 per cent sulfuric acid and 95 per cent glacial acetic acid.

[3] We wish to thank Dr. G. Blix for the generous sample of sialic acid.

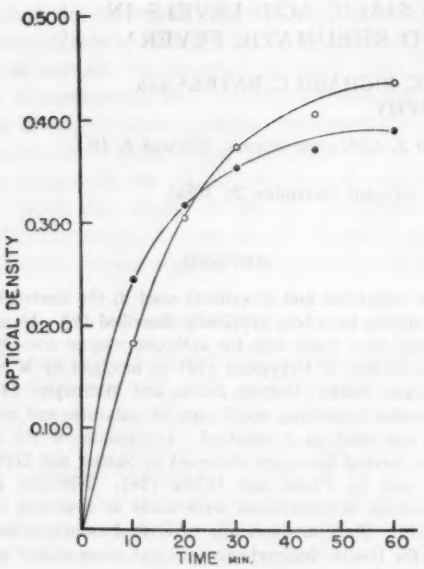

FIG. 1. THE EFFECT OF TIME OF HEATING UPON OPTICAL DENSITY USING A MIXTURE OF 0.4 ML. OF TCA SUPERNATANT SOLUTION AND 5 ML. OF SULFURIC ACID REAGENT

○ Optical density readings measured immediately after cooling and allowing the solutions to come to room temperature.

● Optical density values obtained from the same solutions after standing 24 hours at room temperature.

The effect of time of heating on the color development can be seen in Figure 1. Since in each instance the intensity of the color did not change when the solution was allowed to stand at room temperature for an hour, a 30-minute heating period was selected as convenient for color development. The optical density readings, measured after the heated reaction mixture stood 24 hours at room temperature, have also been plotted in Figure 1.

Aliquots of the supernatant solution varying from 0.2 to 1.0 ml. were heated with 5 ml. of reagent. Within this concentration range a Beer's law relationship obtained.

SUGGESTED PROCEDURE

Reagents

A solution of 10 per cent trichloracetic acid (TCA) was used to precipitate serum proteins.

The 5 per cent sulfuric acid reagent consisted of 5 ml. of concentrated sulfuric acid in 95 ml. of glacial acetic acid.

Method

To 1 ml. of serum in a 15-ml. Pyrex centrifuge tube was added 1 ml. of 10 per cent TCA. The TCA was added slowly with gentle shaking and stirring with a rod to insure complete mixing and fine flocculation of the precipitated protein. The tube was capped with a small beaker and placed in a boiling water bath for 5 minutes. After heating the tube was cooled in an ice bath for 5 minutes. The tube was shaken to remove precipitate adhering to the walls and centrifuged for 5 minutes at 1000 g. in an International Clinical Centrifuge.

Four-tenths ml. of the supernatant solution was transferred to an 18 × 150 mm. rimless Pyrex tube and 5 ml. of the 5 per cent H_2SO_4 reagent was added from a 50-ml. burette. The tubes were capped with 10-ml. beakers and heated in a boiling water bath for exactly 30 minutes, during which time a pink color developed. The solution was cooled in an ice bath for 5 minutes, allowed to come to room temperature, transferred to a calibrated tube and read in a Model 6A Coleman Spectrophotometer at 530 mμ against a 5 per cent H_2SO_4 reagent blank. The results of all determinations have been expressed in terms of the uncorrected optical density. The test was reproducible to the nearest 0.010 optical density units.

The 18 × 150 mm. Pyrex tubes were examined for uniform optical properties. Tubes selected were uniform to ± 0.005 optical density units at an optical density of 0.300 and at a wave-length of 530 mμ. This was well within the standard error of the method. The adaptor on the Model 6A Coleman Spectrophotometer was modified by reducing the vertical light path to 15 mm. in order to accommodate a 5-ml. sample.

CHEMICAL AND PHYSICAL STUDIES

When serum is coagulated by heat, or with acids such as sulfosalicylic, perchloric and trichloracetic, there remains in the filtrate materials (15–18) which do not pass readily through the usual dialysis membranes.

Numerous workers have showed that the concentration of such substances in deproteinized serum was considerably increased in a wide variety of diseases (7). While a large number of methods (7) have been devised for measuring the concentration of these substances in serum very little effort has been directed towards their chemical and physical characterization. Winzler, Burk, and Hesselbach (19) reported that the materials prepared from sulfosalicylic acid filtrates using rat blood contained 6.4 per cent nitrogen and 31.1 per cent carbohydrate. The large carbohydrate (15 per cent) and hexosamine (11 per cent) content of material isolated from human serum by perchloric acid treatment led Winzler, Devor, Mehl, and Smyth to consider these substances to be mucoproteins (20). More recently Weimer, Mehl and Winzler, isolated and characterized a purified mucoprotein (21) which they have designated orosomucoid (7). Schmid (22) independently isolated the same substance.

There is evidence (23–26) that most mucoproteins are altered upon exposure to acid pH values. Under acid conditions apparently, some hydrolysis of the mucoprotein molecule occurs. Amongst the products of hydrolysis were found fragments which contained sialic acid and

which were small enough to pass through the dialysis membrane (23).

The material considered in this report was found in the supernatant solution remaining after the precipitation of serum proteins from hot 5 per cent trichloracetic acid. An equal volume of 10 per cent trichloracetic acid was added with stirring to an aliquot of serum. The mixture was heated for 5 minutes in a boiling water bath and then rapidly cooled to room temperature. The supernatant solution, after removal of the precipitate by centrifugation, was neutralized with 6N NaOH to pH 7. This solution was dialyzed for 48 hours against several changes of water, frozen and lyophilized. An average yield of 38 mg. per 100 ml. of serum was obtained. The material contained approximately 17 per cent hexoses, 3.5 per cent hexosamine, 13 per cent sialic acid and 5.8 per cent nitrogen. An aqueous solution of the product gave an ultraviolet absorption curve sigmoidal in shape without a maximum at 280 mμ. The optical density of the solution was approximately the same throughout the range 250 to 280 mμ. An extinction coefficient ($E^{1\%}_{280}$) of about 2.8 was found. The absorption behavior suggested that the tyrosine and tryptophan content of the material was quite low. The nitrogen content, as well as a positive biuret and xanthoproteic test, indicated the presence of polypeptides.

The material was examined in two different buffer systems in the Tiselius apparatus. A single symmetrical peak which had zero mobility was observed at pH 8.6 in 0.10 ionic strength Veronal buffer. Essentially the same behavior was found at pH 4.8 in 0.10 ionic strength sodium acetate buffer. In the acetate system, in addition to the main peak which did not move, two small peaks with mobilities indicative of a negatively charged molecule were found. The zero mobility of the main component, found at two widely separated pH values, demonstrated that the material isolated as described above carried very little electrical charge. One can conclude, therefore, that a preponderance of the amino acids present in the polypeptide portion of the molecule were of the monoaminomonocarboxy type.

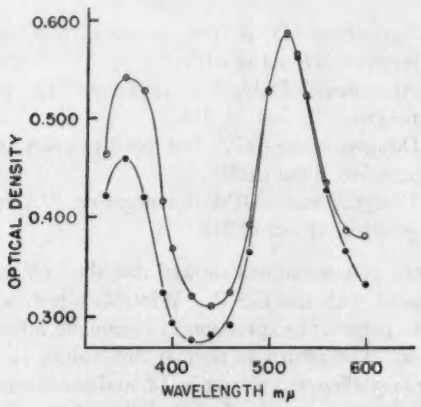

FIG. 2. ABSORPTION CURVES OBTAINED USING DIPHENYLAMINE REAGENT

○ Serum preparation, 1.6 mg. per 1.5 ml. of solution added to 3 ml. reagent.

● Sialic acid, 0.20 mg. per 1.5 ml. of solution added to 3 ml. reagent.

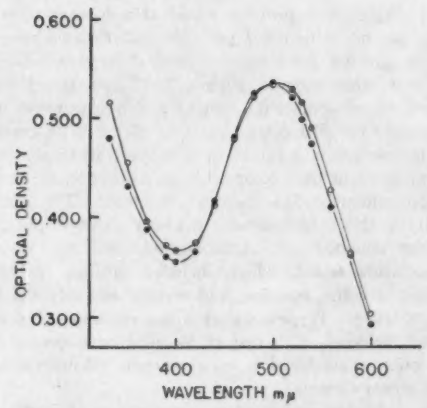

FIG. 3. ABSORPTION CURVES OBTAINED USING 5 PER CENT SULFURIC ACID REAGENT

○ Serum preparation, 3.6 mg. in 0.4 ml. water added to 5 ml. reagent.

● Sialic acid, 0.40 mg. in 0.4 ml. water added to 5 ml. reagent.

The samples used in the above electrophoretic experiments were recovered. The electrolyte was removed by dialysis against water and the samples were lyophilized. The recovered material contained 19 per cent hexoses, 12 per cent nitrogen and 6 per cent hexosamine but no sialic acid. Furthermore, a negative color reaction was obtained when the recovered sample was tested with the 5 per cent sulfuric acid reagent. The marked changes in the chemical composition of the recovered material from that of the original preparation suggested that extensive hydrolysis occurred while the recovered sample was dialyzing against water. A second possibility was that the dimensions of some of the molecules present in the original trichloracetic acid supernatant solution were such that these molecules passed through the Visking casing with difficulty. In the latter case the second period of dialysis was sufficient to remove these molecules.

The material isolated from the trichloracetic acid supernatant solution was tested with diphenylamine reagent and also with the sulfuric acid reagent. The absorption was measured with a model DU Beckman spectrophotometer and may be seen in Figures 2 and 3. The close similarity to the absorption curves obtained when a preparation of sialic acid [3] was tested in a similar manner may be seen in the same figures. This suggested that it was the presence of sialic acid or closely related structures in the serum preparation that was responsible for the color reaction.

All purified mucoproteins tested at a concentration of 6 mg. per ml. with the 5 per cent sulfuric acid reagent gave a positive color reaction and absorption behavior similar to that seen in Figure 3. Those tested were ovomucoid, orosomucoid,[4] fetuin[5] and bovine tonsil mucoprotein. Purified gelatin gave no color. With crystalline bovine serum albumin at a concentration of 6 mg. per ml. a slight pink color with an absorption maximum at approximately 530 mμ was obtained. The optical density in this case however, was only about 10 per cent of that obtained with orosomucoid. Of the various amino acids tested, which included proline, hydroxyproline, histidine, tyrosine, and tryptophan, only the last gave a color. Tryptophan at a concentration of 6 mg. per ml. behaved, with respect to color development and absorption characteristics, in a manner indistinguishable from serum albumin.

The following sugars at a concentration of 5 mg. per ml. gave a negative test: sucrose, fructose, galactose, rhamnose and 2-deoxyglucose. Glucosamine did not produce a color.

Pyrrole and pyrrole derivatives yielded orange colored solutions with absorption maxima at about 480 mμ. With indole-3-acetic acid a pink color and a maximum at 530 mμ in the absorption curve was observed.

The results of the above studies indicated that the reaction may be specific for sialic acid. Although the indole ring reacted with the reagent to give a somewhat similar color the intensity of color developed per unit weight of reactant was much less. For example, 30 μg. of sialic acid produced a color intensity comparable to that given by 6 mg. of tryptophan. In addition, and as discussed above, the indole ring differed from sialic acid with respect to the wavelength at which the absorption maximum occurred.

RESULTS

Normal range

The sialic acid (SiA) test was run on serum from 85 adults and children (male and female) in apparently good health. The normal range of optical density was calculated to be 0.227 to 0.303. This represents two standard deviations (SD ± 0.019) on either side of the arithmetic mean which was 0.265. With the exception of six tests all normal sera fell within this range. Four of the six exceptions fell within three standard deviations. Included in these tests were a group of 16 quiescent rheumatic children ranging in age from 10 to 16 years. These children had not been ill during the previous six months. The normal range for the sialic acid test for these 16 was calculated to be 0.216 to 0.304. The mean was 0.260, and the standard deviation was 0.022.

[4] Dr. Henry Weimer kindly provided the sample of orosomucoid.

[5] We are indebted to Dr. H. F. Deutsch for the sample of fetuin.

TABLE I

The range of SiA values in three diseases

Acute rheumatic fever	(Cases 37)	0.372 to 0.592
Tuberculosis	(Cases 11)	0.292 to 0.665
Cancer	(Cases 6)	0.332 to 0.590

Range for disease states

Both sialic acid and DPA (1) tests were run on aliquots of serum from patients in different stages of pulmonary tuberculosis, tuberculous meningitis, cancer and rheumatic fever. The sialic acid test was elevated during the acute phase of these diseases in all patients as shown in Table I.

Serial determinations were made in parallel with DPA tests on a group of rheumatic fever patients at Great Lakes Naval Training Station. All of these patients were young male adults with typical attacks of rheumatic fever which met Jones' criteria as recently modified (27). The results from the two tests, made on aliquots of serum, were compared. The average optical density reading with the sialic acid test during acute stages of rheumatic fever, as measured on 42 subjects, was 0.465. The results have been divided into four groups:

a. Agreement—DPA test positive, SiA test positive, 178 out of 318.
b. Agreement—DPA test negative, SiA test negative, 95 out of 318.
c. Disagreement—DPA test positive, SiA test negative, 2 out of 318.
d. Disagreement—DPA test negative, SiA test positive, 41 out of 318.

Serial determinations showed that the SiA test, compared with the E.S.R. (Wintrobe) test, was a more persistent expression of rheumatic inflammation. The return to normal SiA values in 18 patients studied was slower in 12, and coincidental in 5. In one case the E.S.R. followed the SiA. The average time difference for a return to normal values was 2 weeks longer for the SiA test than for the E.S.R. (Wintrobe) test. Previous studies (2, 4) showed that the DPA, C-reactive protein, and E.S.R. (Westergren) rise and fall

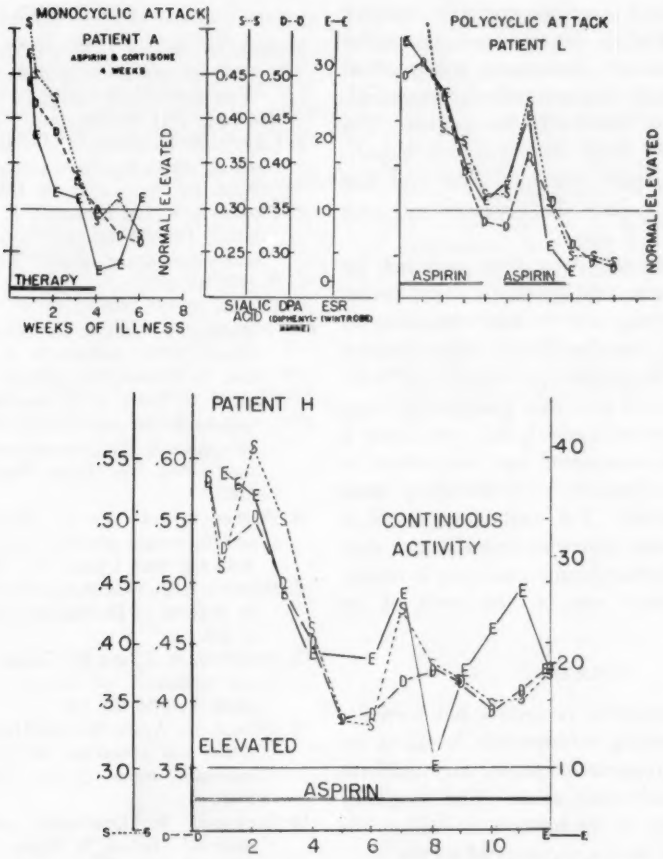

FIG. 4. THE RELATIONSHIP BETWEEN THE SIA, DPA AND E.S.R. DETERMINATIONS DURING THE COURSE OF RHEUMATIC ATTACKS (MONOCYCLIC, POLYCYCLIC AND CONTINUOUS)

S—refers to the sialic acid level
D—indicates the color intensity of the diphenylamine reaction
E—is the sedimentation rate of erythrocytes (Wintrobe).

in parallel. Furthermore, the DPA elevation may continue after the C-reactive protein is negative, and usually persists after a normal level is reached by the E.S.R. (Westergren). The SiA determinations in relation to E.S.R. (Wintrobe) are shown in Figure 4.

DISCUSSION

Evidence has been presented that when serum proteins have been precipitated with hot 5 per cent trichloracetic acid the supernatant solution after dialysis consisted chiefly of uncharged molecules. The substances contained sizable amounts of hexoses, hexosamine and sialic acid, as well as polypeptides. The lability of mucoproteins, when exposed to acids, and the difference in the amount of the material obtained, depending upon time and temperature factors during precipitation, suggested that these substances were probably fragments of those mucoprotein molecules which are normally present in serum.

Additional evidence indicated that these substances were found in elevated amounts in serum samples from a variety of diseases in which serum mucoproteins are known to be elevated (7).

Both chemical and spectrophotometric evidence implicated sialic acid as the structure responsible for color development. Substances tested which did not contain sialic did not produce the characteristic color when heated with the reagent. The material recovered from electrophoretic experiments, which no longer contained sialic acid, lost the capacity to produce the characteristic pink color.

Four other methods have been reported for measuring the sialic acid levels in blood serum (6). These reactions are: 1) Bial's reaction, 2) the direct Ehrlich reaction, 3) the diphenylamine reaction, 4) the tryptophan-perchloric acid reaction (6). Until such time as a quantitative study comparing the various methods has been made it is not possible to recommend any one method as being either quantitatively or qualitatively more reliable than another. The method suggested in this report, although somewhat less sensitive than the Bial and the diphenylamine reactions, is recommended for clinical use on the basis of its simplicity.

SUMMARY

1. A new colorimetric procedure has been described for measuring mucoprotein levels in serum. The only reagents employed are trichloracetic, sulfuric, and acetic acids. The simplicity and the sensitivity of the method, as well as the reproducibility of results, recommend its use.

2. The chromophoric substance was found to be present in the supernatant solution when the serum proteins were precipitated with hot trichloracetic acid. Chemical and physical characterization studies indicated that the substances remaining in the supernatant solution were non-dialyzable, uncharged molecules consisting of monoaminomonocarboxy type polypeptides combined with hexoses, hexosamine and sialic acid.

3. Both chemical and spectrophotometric evidence implicated sialic acid as the structure responsible for the color produced.

4. The optical density values for normal, rheumatic fever, cancer, and tuberculosis sera are reported. Serial determinations on rheumatic fever patients indicated that the method described above, was more sensitive and consistent than the erythrocyte sedimentation rate as an expression of rheumatic activity.

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PHYSICO-CHEMICAL AND IMMUNOLOGIC STUDIES ON MACROGLOBULINS [1]

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Normal sera are resolved in the ultracentrifuge into two major peaks with sedimentation constants of the order of 4.5 and 6.5 S [2] and a minor peak of heavy materials, amounting to less than 3 per cent of the total proteins, with a sedimentation constant of about 20 S.

In 1944, Waldenström (1) reported the presence of a large amount of fast sedimenting moieties (19 to 20 S) in sera of some patients with marked hyperglobulinemia. This finding prompted him to assume that these serum components were of high molecular weight and he named them macroglobulins. He also reported that these serum components could be precipitated out on dilution of the serum with 16 volumes of distilled water.

In recent years a number of workers have reported the presence of components in pathological sera with properties similar to those assigned by Waldenström to macroglobulins (2–10). However, in a number of cases the macroglobulins detected by the precipitation test with distilled water had sedimentation constants as low as 12 S and as high as 30 S. On the basis of electrophoretic mobilities, macroglobulins have been reported to occur either in the β or γ-globulin regions (2, 3, 5, 6, 8–11).

Vogler, Oberhänsli, and Kofler (12) found that the diffusion coefficient for some macroglobulins was 1.2×10^{-7} cm^2. per sec. in contrast to the accepted value for normal γ-globulins of about 3.8×10^{-7} cm^2. per sec. This finding was considered further evidence for the high molecular weight of macroglobulins.

In 1952, Derrien (11) showed that macroglobulins from different pathological sera which possessed similar electrophoretic and ultracentrifugal characteristics nevertheless could be differentiated by their solubility properties. In addition he showed that macroglobulins, apparently homogeneous by electrophoresis, were markedly heterogeneous on the basis of solubility properties.

Recently a number of authors have reported immunologic studies on macroglobulins. Habich (3) concluded that the macroglobulins in some sera did not possess any distinct antigenic groups with respect to normal sera. On the other hand, the macroglobulins of other sera exhibited specific antigenicity. On the basis of immunologic tests with these latter sera, he suggested that some macroglobulins contained group-specific as well as individually specific antigens. Grümer and Klaus (7), using rabbit antiserum to macroglobulin, reported that the macroglobulin serum contained specific antigens not found in normal human serum. Di Guglielmo and Antoninix (2) investigated the immunologic properties of macroglobulins using the anaphylaxis reaction as the criterion for identity of antigens and suggested that macroglobulins possessed individually specific antigenic groups not found in normal human serum.

The amino-acid composition of the macroglobulins has recently been investigated (5–7). The β- and γ-macroglobulins appeared to differ only slightly in their amino-acid content from normal β- and γ-globulins.

During the past two years, we have investigated the sera from four patients with macroglobulinemia. Their case histories have been reported elsewhere (13). The physico-chemical and immunologic results are reported here.

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[2] S refers to Svedberg units.

METHODS

Isolation of macroglobulins

Each of the sera was diluted 16-fold with distilled water. The macroglobulins [3] which precipitated out were centrifuged and the precipitates were washed 3 times with cold distilled water. The precipitates were then dissolved in saline (0.9 per cent NaCl). In attempts at further purification, the macroglobulins of two sera were reprecipitated twice from the saline solution by addition of distilled water. In view of the close similarity of the ultracentrifugal and electrophoretic patterns after the first and third precipitations, the macroglobulins of the other two sera were precipitated only once, thus limiting the inherent losses associated with successive reprecipitations. The macroglobulins in saline solution were Seitz-filtered into sterile vials and stored at 4° C. After precipitation of the macroglobulins, the supernatants were concentrated to the original volumes of the sera by pervaporation in Visking tubing under sterile conditions. The whole sera, the precipitated macroglobulins and the supernatants were compared by electrophoresis, ultracentrifugation and immunologic methods.

Electrophoresis

1) *Free electrophoresis.* All samples were examined in a Spinco model H Tiselius apparatus at 0.8° C, using veronal buffer at pH 8.6 and ionic strength 0.1. The solutions were dialyzed through Visking tubing against the buffer for a period of 24 to 36 hours prior to electrophoretic analyses. The protein concentration of each sample was about 1 per cent as determined refractometrically (14). The macroglobulin fraction of one serum (A.B.) was analyzed in acetate buffer at pH 3.65 and 4.8, phosphate buffer at 6.5, 7.0, and 7.7 and veronal buffer at pH 8.6. The ionic strength of all buffers was 0.1.

2) *Paper electrophoresis.* All samples were investigated by paper electrophoresis, according to a procedure described previously (15). Veronal buffer (pH 8.6, ionic strength 0.1) was used in this study. After electrophoresis the papers were stained for proteins and carbohydrates with Amido black 10B (16) and the periodic acid Schiff reagent (17), respectively.

3) *Starch electrophoresis.* One serum (A.B.) was separated into its electrophoretically distinct components using starch electrophoresis. The method is fully described elsewhere (18). The starch block was divided into segments according to the protein distribution curve as shown in Figure 5. The serum protein fractions were eluted from the starch block and examined in the ultracentrifuge and by paper electrophoresis.

As shown in Figure 5, the γ_2-globulins were divided into four sub-fractions in an attempt to delimit more precisely the locale of the macroglobulins.

[3] In this paper the term macroglobulins refers to serum components precipitated by 16-fold dilution of pathological serum with distilled water. This treatment did not cause precipitation of the generally found fast sedimenting component of normal serum ($S \simeq 20$).

Ultracentrifugation

The Spinco model E optical ultracentrifuge was used to determine the sedimentation constants of the proteins in different samples. The solutions were made up in or were diluted with saline. The average temperature during centrifugation was 19 to 20° C and the rotor speed was 59,780 r.p.m. The sedimentation constants were not recalculated for standard conditions. The effect of protein concentration on sedimentation constants was not investigated in view of the apparent complexity of these materials.

Immunologic methods

Albino rabbits of both sexes (4 to 8 kg. body weight) were immunized with the following antigens: 1) the macroglobulin fraction of serum A.B. (1.5 per cent in saline), referred to hereafter as M, 2) undiluted pooled normal human serum referred to as NHS, 3) Squibb gamma globulins [4] (3.5 per cent in saline) referred to as GG. One-ml. aliquots of each antigen solution were injected intravenously into two rabbits three times a week (on 3 successive days) for a period of four to five weeks. The animals were bled 6 to 9 days after the last injection. The antisera to each antigen were pooled, Seitz-filtered into sterile bottles and stored at 4° C. The antisera were tested for presence of antibody by ring test.

To determine the degree of antigenic similarity between M and GG, the following experiment was carried out (experiment A). In a series of 19 tubes, 1-ml. aliquots of the antiserum to M, diluted fourfold, were mixed with equal volumes of a solution of GG in halving dilutions. The maximum concentration of the GG solution used was 9 per cent. Incubation of the tubes for 2 hours at 37° C followed by incubation for 48 hours at 4° C resulted in the formation of visible precipitates. The tubes were then centrifuged and aliquots of the supernatants were checked by ring test for excess antibody with GG and for excess of antigen with anti-M serum. The supernatants containing excess antibody were further absorbed with GG until ring tests were negative with GG. These latter supernatants were then tested with a solution of M for the presence of specific antibody.

As a further test for the specific antigenicity of macroglobulins the following experiment was performed (experiment B). To a 3-ml. aliquot of anti-M serum, GG was added in sufficiently high concentration to inhibit any precipitation (45 ml. of 2.5 per cent solution) between GG and anti-M serum. In a series of 12 tubes, 1-ml. aliquots of this solution were incubated with equal volumes of M in halving dilutions, in concentrations varying from 15 mg. per ml. to 8 γ per ml. In a series of control tubes GG was substituted for M. In another experiment the procedure was repeated using, instead of GG, a solution of concentrated serum proteins (20 per cent in saline), isolated from normal human serum by precipitation with ammonium sulphate at 66 per cent saturation.

[4] Batch 88 Squibb Co., obtained through the courtesy of the American National Red Cross, Washington, D. C.

Agar gel techniques

To determine the minimum number of antigenic moieties in each antigen solution, the degree of cross-reactivity of the various systems and the presence of specific antigenic groups or antigens in the macroglobulin fraction, the following agar gel techniques were used.

Oudin tubes were set up for "single diffusion" (19). The antisera were diluted with 3 volumes of a 1 per cent solution made up in saline. All the antigen solutions used in these experiments were made up to 2 per cent in saline. Each antiserum was tested with the 3 antigen solutions: M, NHS, and GG.

The method recently described by Oudin (20) using glass cells with parallel walls was used to determine the antigenic similarities of M, GG and NHS. In these experiments the antisera and antigen solutions were diluted with equal volumes of 2 per cent agar. The concentrations of the GG, M and NHS were 1.8 per cent, 1.5 per cent and 3.5 per cent, respectively. The lower section of the cell was filled with agar solution containing the antiserum, the central section with agar only, and each of the two halves of the upper section with one of the antigens in agar (see Figure 7).

In order to ascertain further whether the macroglobulin fraction contained any specific antigenic moieties not present in normal serum, an experiment similar in principle to experiment B (described above) was devised. A solution of the normal serum proteins was made up to a concentration of 20 per cent in the agar used for the Oudin double diffusion method. This solution, referred to hereafter as AP, was used instead of pure agar in all compartments of the Oudin cell for "double diffusion." Thus a uniform concentration of normal serum proteins was maintained throughout the whole cell. Anti-M serum in AP was placed in the lower compartment and M in AP was placed in the antigen compartment.

In a control cell the upper section was divided into three segments (Figure 8B). Segments I and II were filled with solutions of macroglobulins in AP, the concentrations of M being 0.75 per cent and 0.38 per cent, respectively. Segment III contained only AP solution.

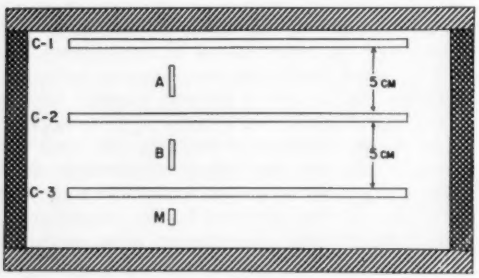

FIG. 1. IMMUNO-ELECTROPHORESIS

A and B represent ditches containing the antigen solution to be separated; C-1, C-2 and C-3 represent channels containing the antisera; M represents the ditch for the "marker" solution.

Ouchterlony plates (19) with four circular wells symmetrically placed with respect to each other in a Petri dish of 9-cm. diameter were used. Two wells, diametrically opposed, were filled with anti-M and anti-GG, and the two other wells with the homologous antigens. The concentrations of the reactants were the same as in the previous experiment.

Immuno-electrophoresis was performed according to the method of Williams and Grabar (21). The glass plates used were 30 cm. × 15 cm. and were covered with transparent films of agar, as described by Oudin (22). Electrophoresis was carried out in an agar medium (1 per cent agar in veronal buffer at pH 8.6 and ionic strength 0.025). The height of the agar layer was 3 mm. In view of the high degree of electro-osmosis in agar, the material to be separated was applied in most experiments, in small ditches (20 mm. × 4 mm. × 3 mm.) 10 cm. from the anode (A and B in Figure 1). Two antigen solutions made up in agar[5] were separated simultaneously on each plate and after electrophoresis the antisera in agar were placed into 5 mm. wide channels (C) which were cut longitudinally parallel to the direction of electrophoretic separation (Figure 1). To follow visually the electrophoretic separation a "marker" (M) was used. This consisted of normal serum mixed with small amounts of bromphenol blue (travelling slightly ahead of the albumin) and hemoglobin (travelling with the β-globulins.) The electrophoresis was carried out in a well-insulated box to minimize evaporation. The electrode vessels were identical to those used for paper electrophoresis. The voltage applied across the plate was 30 to 45 volts and the current was about 20 mA. The duration of an experiment was 24 to 30 hours during which time the albumin and γ-globulin were separated over a distance of 15 cm. The distribution of the separated proteins was established with the help of a "print," taken as described for starch electrophoresis (18). Immediately after filling channels C-1, C-2 and C-3, the agar plates were covered with glass plates and sealed with silicone grease. The plates were then placed on a level surface at room temperature and observed intermittently for the formation of precipitin bands.

RESULTS

Free electrophoresis

The results of free electrophoresis for the four sera and for the respective supernatants (serum minus macroglobulins) are given in Table I together with the results obtained in this laboratory for normal sera. As can be seen the γ-globulin fractions of all four sera were highly elevated. However, only the first three sera contained appreciable quantities of macroglobulins. The mac-

[5] Two volumes of the antigen solution were mixed with one volume of a 3 per cent agar solution in veronal buffer of ionic strength 0.075.

TABLE I

Free electrophoresis

Case		Relative percentages					Gm. per 100 ml. in whole serum	
			Globulins					
		Albumin	Alpha-1	Alpha-2	Beta	Gamma	Total protein‡	Macro-globulins§
C. V. (Female)	W*	33.1	8.8	19.8	11.8	26.5	7.38	2
N. R. (Male)	W	16.8	4.8	7.2	12.7	58.5	10.68	5
	S	41.4	10.2	5.9	9.6	32.9		
A. B. (Female)	W	26.8	3.6	7.1	8.9	53.6	16.10	8
	S	61.6	5.0	12.7	12.6	7.1		
L. S. (Female)	W	31.3	3.2	7.0	9.2	49.3	12.18	0.7
	S	34.0	3.4	7.0	9.2	46.4		
Normal† human serum	Male	59.4 ± 3.2	4.7 ± 1.8	8.6 ± 1.4	12.9 ± 1.3	14.3 ± 2.5	8.12 ± 0.59	
	Female	58.4 ± 2.3	5.2 ± 0.7	9.8 ± 1.0	12.4 ± 1.2	14.2 ± 1.8	7.92 ± 0.71	

* W—Whole unfractionated serum, S—Supernatant (serum minus macroglobulins).
† Average values as found for 22 male and 14 female sera ± one standard deviation.
‡ Determined refractometrically.
§ Approximate values.

roglobulin fraction of each of three sera (C. V., A. B. and L. S.) gave rise to single symmetrical peaks with mobility values of 0.64, 1.10 and 0.79 cm.^{2} per volt per sec., all of which fell below the range of those for normal γ-globulins (1.23 to 1.67 cm.^{2} per volt per sec.) The macroglobulin fraction of the fourth serum (N.R.) resolved itself into three peaks with mobilities (1.12, 3.29, 4.70 cm.^{2} per volt per sec.) corresponding to those of γ-, β- and α_2-globulins. The macroglobulin fraction of the serum A.B. could not be resolved by free electrophoresis into more than one peak within the pH range 3.6 to 8.6. Figure 2 represents the patterns obtained by free electrophoresis for the whole sera A.B. and N.R. and their macroglobulins and supernatants.

Paper electrophoresis

The results of paper electrophoresis confirmed those obtained by free electrophoresis. Staining of the electrophoretograms of the whole sera with fuchsin revealed, in addition to the fuchsin stainable bands found in normal sera, the presence of material rich in carbohydrate in the γ-globulin region. The fuchsin stainable band in the γ-globulin region appeared to be associated with the macroglobulins, as evidenced by its presence on the electrophoretograms of the macroglobulin solutions and its absence in the supernatants.

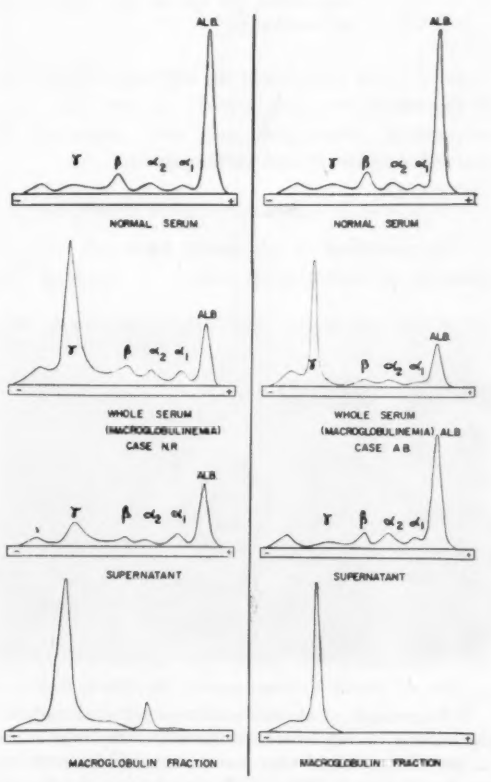

FIG. 2. SEPARATION BY FREE ELECTROPHORESIS

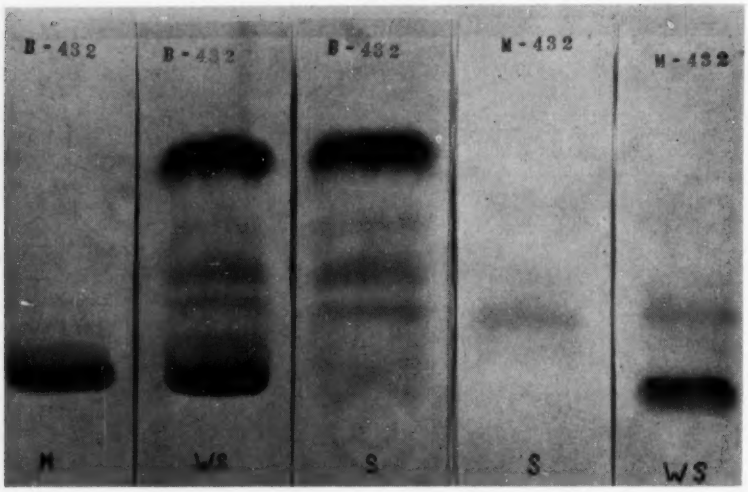

FIG. 3. PAPER ELECTROPHORESIS OF SERUM A.B.

WS represents whole serum; M represents macroglobulin fraction; S represents supernatant. The three strips on the left are stained with Amido-black 10B for proteins, while the two on the right are stained with periodic acid Schiff reagent for carbohydrates.

Figures 3 and 4 represent the electrophoretograms of the whole sera of A.B. and N.R. and their corresponding macroglobulins and supernatants stained for protein and carbohydrate.

Separation of A.B. serum by starch electrophoresis

The resolution of this serum by starch electrophoresis is illustrated in Figure 5. Each of the eluted fractions was examined by both paper electrophoresis and ultracentrifugation. Four of the eluted fractions, albumin, α_1-, α_2- and β-globulins, displayed normal migratory and staining properties by paper electrophoresis. Ultracentrifugation revealed a rapidly sedimenting material (16.4 S) in low concentration associated with the α_2 fraction. The four γ-globulin sub-fractions were all heterogeneous in the ultracentrifuge. Three of the latter fractions (B, C, D,) were composed of normal and macroglobulin components. Only one fraction (A) appeared to be free of slowly sedi-

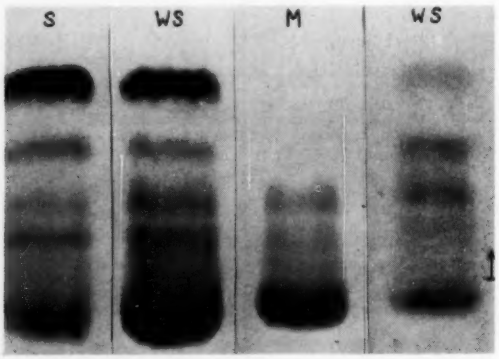

FIG. 4. PAPER ELECTROPHORESIS OF SERUM N.R.

WS represents whole serum; M represents macroglobulin fraction; S represents supernatant. The three strips on the left are stained with Amido-black 10B for proteins, the fourth strip is stained with periodic acid Schiff reagent for carbohydrates.

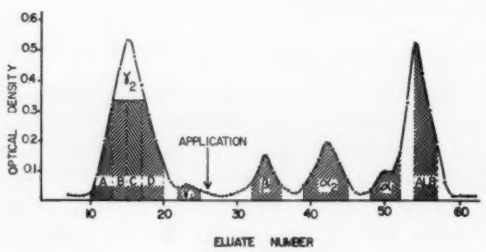

FIG. 5. SEPARATION OF SERUM A.B. BY STARCH ELECTROPHORESIS

The shaded areas represent fractions eluted from the starch block. A, B, C and D represent subfractions of γ_2-globulins.

TABLE II

Sedimentation constants of serum fractions obtained by starch electrophoresis (serum A.B.)

Gamma-2								
A	B	C	D	Gamma-1	Beta	Alpha-2	Alpha-1	Albumin
	7.3	6.5	6.5	7.5	4.8	6.7	3.7	4.0
16.4	15.2	15.7	14.6			16.5		
23.2	21.7	22.0	22.2					
	28.8	28.1	28.8					

menting γ-globulin (Table II). The data of Table II would suggest that the macroglobulins migrate at a somewhat slower rate in an electric field at pH 8.6 than do the γ-globulin constituents regularly found in normal serum.

Ultracentrifugation

The macroglobulin fractions of the four sera possessed components of high sedimentation constants. The results of ultracentrifugal analyses are presented in Table III. The macroglobulin fraction of serum N.R. resolved itself into a broad spectrum of components with sedimentation constants of 3.4, 6.0, 11.0, 12.6, 17.7 and 27.5 S. By far the greater part of the macroglobulin fraction (82 per cent) was composed of the fast sedimenting material (peaks with S values of 11 to 27.5). The macroglobulins in serum A.B. (Figure 6) also appeared to be heterogeneous ultracentrifugally, possessing sedimentation constants of 16.4, 23.3, 28.8 S, in addition to a small peak with sedimentation constant of 7.4 S. These values correspond to the S values determined for the four γ-globulin subfractions isolated by starch electrophoresis. The macroglobulins of serum L.S. had sedimentation constants of 6.5, 10.6 and 15.3 S. Sedimentation constants of 6.5, 18.5 and 26.0 were calculated for the components in the macroglobulin fraction of serum C.V.

TABLE III

Ultracentrifugal analysis of the macroglobulins

Case	Sedimentation constants*	Percentages†
C. V.	6.5	55.0
	18.5, 26.0	45.0
N. R.	3.4	5.8
	6.0	11.9
	11.0	11.3
	12.6, 17.7	61.2
	27.5	9.8
A. B.	7.4	4.6
	16.4	56.5
	23.3	32.2
	28.8	6.7
L. S.	6.5	57.5
	10.6	22.4
	15.3	20.1

* In Svedberg units.
† Relative distribution of the components in the macroglobulin fractions.

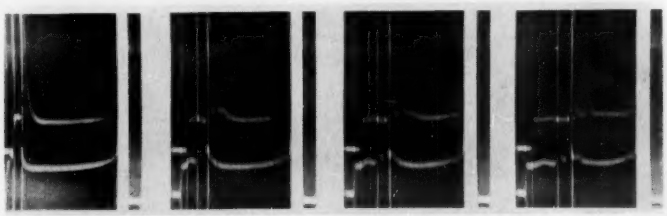

FIG. 6. ULTRACENTRIFUGATION OF SERUM A.B.

The lower pattern represents macroglobulins in 1:2 dilution analyzed in a standard cell, the upper pattern represents macroglobulins in 1:3 dilution in a cell provided with a wedge disc. The above frames (from left to right) were photographed at 10, 17, 22 and 28 min. after the rotor attained full speed of 59,780 r.p.m.

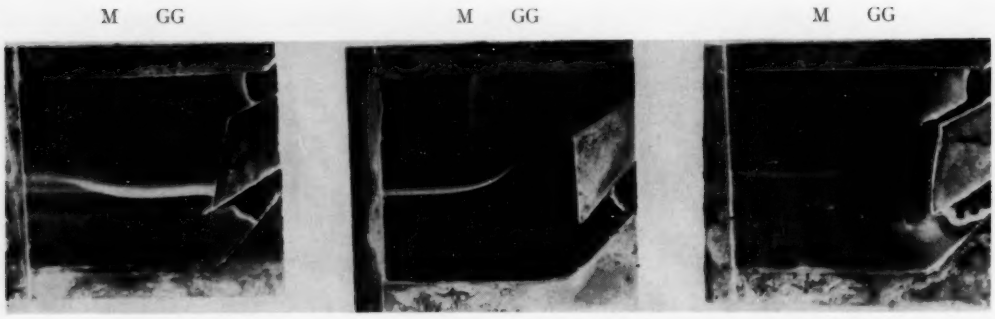

FIG. 7. DOUBLE DIFFUSION EXPERIMENTS IN OUDIN CELLS WITH PARALLEL WALLS

M represents macroglobulin fraction of serum A.B., GG represents γ-globulin; Anti-M represents rabbit anti-macroglobulin serum; Anti-GG represents rabbit anti-γ-globulin serum; Anti-NHS represents rabbit anti-normal human serum.

Immunologic results

1. *Precipitin method*—In experiment A precipitation occurred in tubes 2 to 18 in the series of 19 tubes. Supernatants from tubes 2 to 18 gave positive ring tests with GG and those from tubes 1 to 12 gave positive ring tests with anti-M serum. The supernatants were subsequently absorbed with GG until antibody could no longer be detected against GG. However, the supernatants still gave positive ring tests with M, thus demonstrating the presence of a precipitating antibody-antigen system specific to M.

In experiment B, precipitates were formed only in tubes 5 to 10 of the series of 12 tubes. The optimum zone appeared to be in tube 8. Similar results were obtained when the proteins of normal serum were substituted for GG, the optimal zone being observed in tube 9.

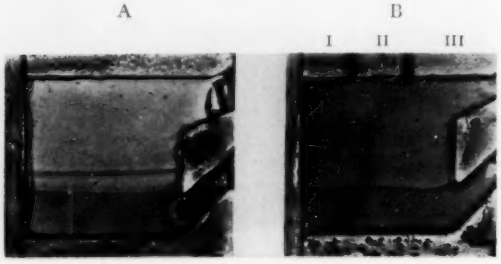

FIG. 8. DOUBLE DIFFUSION EXPERIMENTS IN OUDIN CELLS WITH PARALLEL WALLS

The cells represent the system of macroglobulins with rabbit anti-macroglobulin serum in presence of an excess of normal serum proteins.

Agar gel techniques

Precipitin bands were formed in all Oudin tubes indicating thus that M, GG and NHS contained some common antigenic moieties.

The results of the experiments using the Oudin cells with parallel walls (Figure 7) demonstrate the extent of antigenic similarity of M and GG.[6] In Figure 7A it appears that both M and GG contained at least three identical antigenic moieties. In plate 7B seven bands were formed between M and anti-M, two of which were common to the GG-anti-M system. As revealed in Figure 7C a minimum of four bands was formed only between M and anti-NHS and three additional bands were common to the two systems. Figure 8 illustrates the results for the M-anti-M system in agar in the presence of an excess of normal serum proteins. As can be seen in Figure 8A one distinct band was produced across the central portion of the cell when the upper section was filled completely with macroglobulin in AP solution. This result indicates the presence of at least one specific antigenic moiety in the macroglobulin fraction which is absent from normal serum. This conclusion is further supported by the discontinuity of the band

[6] The bands were much more clearly delineated in the agar than in the photographic reproductions.

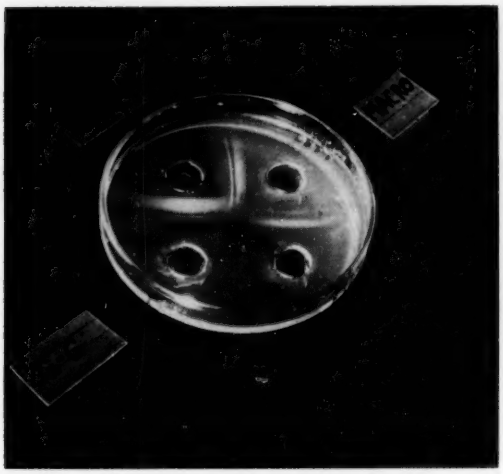

FIG. 9. OUCHTERLONY PLATE

Macro represents macroglobulin fraction of serum A.B., GG represents γ-globulin; Anti-macro represents rabbit anti-macroglobulin serum; Anti-GG represents rabbit anti-γ-globulin serum.

in the region containing only the AP solution in the control experiment (Figure 8B).

The Ouchterlony plate is shown in Figure 9. M versus anti-GG gave rise to four bands while each of the homologous antigen-antibody systems gave rise to two bands only. GG gave only a faint band against anti-M.

The immuno-electrophoretic results are illustrated in Figures 10 and 11.

Electrophoresis of the NHS in agar followed by the application of the anti-M, anti-NHS and anti-GG sera in the longitudinal channels resulted in the formation of a large number of precipitin bands (Figure 10). The anti-GG reacted with the electrophoretically separated NHS proteins to yield a long continuous precipitin band extending from the γ-globulin region into the albumin region, in addition to two faint bands in the β-globulin and albumin regions. The long precipitin band was also formed by the NHS anti-NHS system but not by the NHS anti-M system. In all other respects, the anti-M and anti-NHS appeared to form the same number of bands similar in their distribution. The absence of bands between anti-M and NHS (in Figure 10) and between anti-M and GG (in Figure 11) in the region of γ-globulins is somewhat perplexing in view of the copious precipitates obtained when anti-M was incubated with GG as previously mentioned. When M was separated by electrophoresis in the agar and anti-M

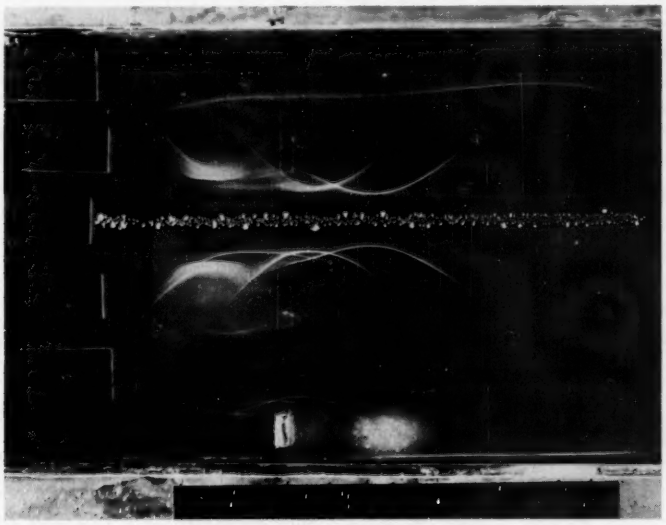

FIG. 10. IMMUNO-ELECTROPHORESIS

NHS represents pooled normal human serum; Anti-GG represents rabbit anti-γ-globulin serum; Anti-NHS represents rabbit anti-normal human serum; Anti-Macro represents rabbit anti-macroglobulin serum.

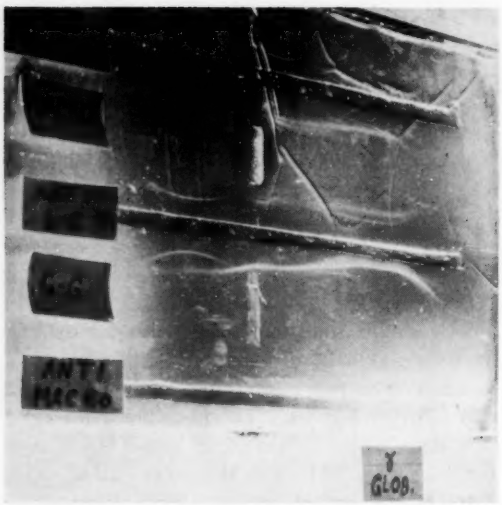

FIG. 11. IMMUNO-ELECTROPHORESIS

Macro represents macroglobulin fraction of serum A.B.; GG represents γ-globulin; Anti-Macro represents rabbit anti-macroglobulin serum; Anti-GG represents rabbit anti-γ-globulin serum.

and anti-GG were deposited into the lateral channels, bands resulted in the γ-globulin region with the anti-M serum and in the albumin, α- and γ-globulin regions with the anti-GG serum. On the other hand, a long continuous heavy band was formed between the electrophoretically separated GG and anti-GG which extended from the γ-globulin region into the albumin region. In addition, a small, faint but distinct band was detected in the albumin region.

One of the difficulties encountered during the immuno-electrophoretic experiments was the low solubility of the macroglobulins in the buffer used for the immuno-electrophoresis. The macroglobulins (A.B.) were essentially insoluble in veronal buffer of ionic strength 0.025 and pH 8.6. However, the use of veronal buffer of higher ionic strength, in which the macroglobulins were soluble, resulted in the passage of higher current, accompanied by increased evaporation of water from the agar. This effect resulted in deformation of the agar gel. Therefore, veronal buffer of ionic strength 0.025 was resorted to even though some elements of the macroglobulin solution precipitated about the zone of application.

DISCUSSION

Although the macroglobulins of three sera gave rise to only single symmetrical peaks in the Tiselius cell and appeared to be slow moving γ-globulins, ultracentrifugal analysis demonstrated the high degree of heterogeneity of these fractions. The macroglobulins of the A.B. serum could not be resolved by free electrophoresis at different pH's into more than one component. However, by ultracentrifugation this fraction was shown to contain at least four easily distinguishable components (S values of 7.4, 16.4, 23.3 and 28.8 S). The macroglobulins of only one serum (N.R.) were resolved by free electrophoresis into three components. This to our knowledge is the first case displaying electrophoretically heterogeneous macroglobulins.

The results of paper electrophoresis showed clearly the presence of carbohydrate rich material in the macroglobulin fraction as revealed by the Schiff reagent. In general, only a faintly visible carbohydrate band is detectable in the γ-globulin region of normal sera.

Recently, Wallenius, Trautman, Franklin, and Kunkel (23) reported that the "19 S" heavy components normally found in human serum were associated with the α_2-globulins (65 per cent) and with β- and γ-globulins (35 per cent). In addition they found small amounts of heavier components (28 S and 44 S) to be distributed throughout the γ-globulin region. The results of our ultracentrifugal analyses of the fractions of serum A.B., isolated by starch electrophoresis, confirm in part the results of the above workers. As illustrated in Table II the α_2-globulins of serum A.B. which were not water precipitable contained a heavy component with a sedimentation constant of about 16.5 S. Heavy components were also found throughout the entire γ_2-globulin region as in the experiments of other workers (23) but were absent in the γ_1-globulin region. Moreover, the electrophoretically slowest components of γ_2-globulins appeared to contain only fast sedimenting materials (fraction A of Table II). Thus, on the basis of our physico-chemical results, it is not possible to arrive at a clear-cut conclusion whether macroglobulins are distinct serum moieties synthesized during the course of the disease (Waldenström syndrome) or whether they are the result

of a greatly accelerated synthesis of a moiety present in normal human serum in only negligible amounts.

On the other hand, the immunologic results tend to indicate that macroglobulins contain specific antigens (or at least specific antigenic groups) not present in normal serum. The fact that anti-M serum gave positive ring tests with NHS, GG and M demonstrates that the material precipitated with distilled water from the sera containing macroglobulins possessed at least some antigens identical to those found in normal human serum. As no single zone of optimal proportions was detected in the precipitin test between anti-M serum and GG, it can be assumed that both M and GG are multiantigenic systems. The findings that precipitation occurred over a wide range of antigen concentrations (from 45 mg. per ml. to 0.6 γ per ml.) support this interpretation.

In an attempt to demonstrate the presence of antigenically specific moieties in M, experiment B was performed. As is generally known, specific precipitation in a solution containing a number of antigen and antibody systems can be prevented by having all the antigens in great excess. In experiment B, anti-M serum was mixed with a sufficiently high excess of GG to inhibit precipitation. Titration of this solution with M resulted in precipitation occurring only in the six central tubes of the precipitin series of 12 tubes. This precipitation can be attributed to the presence of an antigenic factor or factors present in the macroglobulins and absent in the γ-globulin preparation used.

However, the possibility still existed that this factor might have been associated with any of the other normal serum protein fractions. A concentrated solution of normal serum proteins was substituted for the γ-globulins in the above experiment. As previously stated, no precipitation occurred between the anti-M serum when incubated with a concentrated solution of normal serum proteins. Titration of this solution with M resulted in precipitation occurring only in six tubes, the Dean and Webb optimal zone being only one tube removed from that obtained with GG. Therefore, the precipitation which occurred on addition of the macroglobulin solution to the anti-M serum in presence of an excess of GG or normal serum proteins must be attributed to some antigenically specific entity associated with the macroglobulins.

The Oudin technique, using the cell with parallel walls, allows one to demonstrate the presence of identical antigens and/or antibodies in different solutions. The results obtained with this technique (multiplicity of bands in the systems illustrated in Figure 7) confirm the conclusion arrived at on the basis of the classical precipitin method that both M and GG are complex systems containing several immunologically identical antigens and possibly some specific antigens as well. Since a precipitin band was obtained with the M-anti-M system in the presence of an excess of normal serum proteins (Figure 8), one could conclude that our macroglobulin fraction contained some antigenically specific material absent from normal serum. In essence, these results corroborate the results of Experiment B.

Immuno-electrophoresis was used in the hope that it would reveal whether the different antigens present in M solution were associated with distinct serum proteins separable by electrophoresis. The results of Figure 10 (NHS-anti-M system) would suggest that M possessed most of the antigens present in normal serum. On the other hand the formation of the bands in the γ-globulin region of Figure 11 between M and anti-M confirms the results of the ultracentrifugal analyses of the fractions obtained by starch electrophoresis, demonstrating that the water precipitable heavy component was principally associated with γ-globulins. The formation of additional bands in the region of the albumin and α-globulins in Figure 11 between M and anti-GG on the one hand and GG and anti-GG on the other would suggest that both M and GG contained trace amounts of some antigenic constituents having mobilities of albumin and α-globulins in addition to moieties with properties similar to those of γ-globulins. These results are noteworthy as neither paper electrophoresis nor free electrophoresis of the M or GG revealed the presence of any albumin or α-globulin components, and would thus confirm the claims of Oudin and Grabar (20, 21) that the use of gel technique allows for the detection of minute amounts of antigen or antibody.

The immuno-electrophoresis of NHS in agar yields with anti-NHS and anti-GG a number of

short bands in addition to a long continuous band extending from the γ-globulin region into the albumin region (Figure 10). This band is most pronounced in the γ-globulin region. This result can be explained in two ways. It may be due to cross-reactivity between GG and all the other proteins normally found in serum. This would imply that the other serum proteins contain some antigenic groups identical to those of γ-globulins. The other explanation would be that γ-globulins are preferentially absorbed onto the agar matrix during their migration by electro-osmosis from the zone of application to their final location on the agar plate.

On the basis of the immuno-electrophoretic results obtained with the GG-anti-GG system (Figure 11) it would appear that the first hypothesis might be ruled out. For, as can be seen in Figure 11 a long continuous band similar to the bands obtained with the NHS-anti-NHS or anti-GG system (Figure 10) was also formed between the γ-globulins and anti-GG. This band also extended from the γ-globulin region into the albumin region. However, in view of the contradictory results obtained recently by Slater (24) who claims to have detected some antibody titer associated with β- and α-globulin in addition to the bulk of the antibody residing in γ-globulins, both factors, *viz.*, cross-reactivity of the different serum proteins and absorption, might be partly responsible for the formation of the precipitin band throughout almost all of the protein spectrum. A further point that deserves mention is the reliability of the agar gel techniques in detecting combination between antigen and antibody. Since the anti-M and anti-GG sera formed several clearly delineated bands with either of the two antigens (M or GG) in the Oudin cells (Figure 7), and since copious precipitates were obtained on incubating anti-M with GG in saline, the appearance of only faint lines by immuno-electrophoresis for the anti-M and GG system (Figure 11) is somewhat perplexing. Similarly, only faint bands were formed between anti-M and GG in the single diffusion Oudin tubes and on the Ouchterlony plate (Figure 9). It should be emphasized that although agar gel techniques will, in general, detect trace amounts of antigen or antibody in some systems, in other cases—for reasons which we do not know as yet—almost complete inhibition of precipitin bands may occur.

SUMMARY

Sera from four cases of macroglobulinemia (Waldenström syndrome) were investigated by means of zone (paper and starch) and free electrophoresis, ultracentrifugation and immunologic methods (precipitin technique and the Oudin, Ouchterlony, and Grabar agar gel technique).

On free electrophoresis the macroglobulin fraction of three sera gave rise to single symmetrical peaks with mobility of a slow moving γ-globulin, while the macroglobulin fraction of the fourth serum was resolved into three peaks with mobilities of α_2-, β- and γ-globulins.

The paper electrophoretograms revealed the presence of material rich in carbohydrate associated with the macroglobulin fractions.

The ultracentrifugal analyses demonstrated that the macroglobulin fractions were heterogeneous, the sedimentation constants of the different constituents varying from 10.6 to 28.8 S.

The immunologic methods indicated the presence of antigenically specific material in the macroglobulin fraction which is absent from normal serum.

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A STUDY IN DOGS OF METHODS SUITABLE FOR ESTIMATING THE RATE OF MYOCARDIAL UPTAKE OF RB^{86} IN MAN, AND THE EFFECT OF L-NOREPINEPHRINE AND PITRESSIN® ON RB^{86} UPTAKE [1]

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These experiments were performed to test the accuracy of techniques which are suitable for estimating the rate of uptake of Rb^{86} by the myocardium in man. In the experimental animal it was possible to compare estimates that were based on external measurements of the type possible in man with the actual myocardial uptake of Rb^{86} determined by direct analysis of the heart after sacrifice. The rate of myocardial uptake of Rb^{86} was of interest because factors which influence it, such as the rate of coronary blood flow and the permeability of capillaries and muscle fibers, probably likewise affect the uptake of important metabolites by the heart. Since rubidium resembles potassium in its biological behavior, it might be possible to extrapolate to any gross changes in the rate of potassium uptake or concentration occurring with heart disease. As a partial test of the relationship between coronary blood flow and Rb^{86} uptake rate in the heart, l-norepinephrine or Pitressin® were administered intravenously to several dogs because of the known effects of these drugs on the coronary blood flow (1, 2). If an estimate of coronary blood flow could be obtained in intact man without catheterization of the coronary sinus, the variations in coronary blood flow in large numbers of normal subjects and patients with various types of cardiac disease could be studied. $Rubidium^{86}$, which has a 1.1 mev. gamma emission and a $T\frac{1}{2}$ of 19.5 days, has been used as if it were a tracer of potassium since the 12.4-hour $T\frac{1}{2}$ of K^{42} makes its use difficult.

$Rubidium^{86}$ is not actually a tracer of potassium (3). However, rubidium resembles potassium chemically, and has biologic effects on the heart similar to those produced by potassium (4–7). These two elements are partitioned between the myocardium and plasma in almost identical ratios, and the rates of uptake of each by the various organs of the dog are qualitatively similar (8). The rates of uptake of K^{42} and Rb^{86} by the human erythrocyte *in vitro* have been found to be very nearly the same (9). The processes involved in bringing about uptake of the two elements seem to be similar in this type of cell at least, since factors reducing K^{42} uptake, such as cooling, increase in plasma potassium concentration, and the addition of iodoacetate to the plasma, produce a proportional reduction in Rb^{86} uptake (10).

The general procedure in these experiments was to maintain a nearly constant concentration of Rb^{86} in arterial blood by the continuous injection of isotope, usually for 30 minutes, while an indication of the rise of myocardial Rb^{86} concentration was obtained from a collimated, recording scintillation ratemeter placed over the precordium. From these data the turnover rate of myocardial potassium, which is defined as the fraction of myocardial potassium exchanging with the plasma per minute, and the amount of plasma cleared of Rb^{86} by 100 Gm. of myocardium in one minute were calculated using certain simplifying assumptions. The reliability of these estimates was evaluated by comparing them with the results of direct analysis of the myocardium after sacrifice.

MATERIALS AND METHODS

Mongrel dogs weighing 6.7 to 14.3 Kg. (mean 10.7 Kg.) were anesthetized with 30 mg. sodium pentobarbital per Kg. intravenously and taped to a frame so constructed that they could be held securely in a prone position over the precordial monitor. Supplementary anesthesia of 30 to 60 mg. sodium pentobarbital was occasionally necessary. Under fluoroscopic control, the frame was adjusted so that the approximate center of

[1] Supported by the R. A. Billups Fund for Research in Heart Disease and aided by a U. S. Public Health Service Grant, H-143.

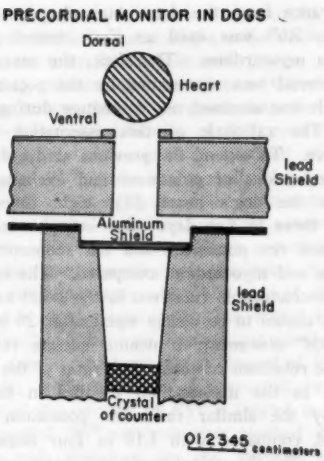

FIG. 1. SCHEMATIC REPRESENTATION OF THE ARRANGEMENT OF THE PRECORDIAL MONITOR IN PRONE, ANESTHETIZED DOGS

the ventricular mass coincided with the vertical axis of the counter crystal. After intravenous injection of 100 to 150 mg. heparin, frequent recordings of pulse and mean femoral arterial blood pressure were begun using a mercury manometer. The mean arterial blood pressure averaged 140 mm. Hg in dogs not receiving drugs, and the mean pulse rate was 160 per minute. Blood from the opposite femoral artery was allowed to flow through 300 cm. of 1.7 mm. internal diameter polyethylene tubing and returned to the femoral vein. A drop bottle was interposed, and 60 cm. of this tubing was wrapped around a probe-type Geiger-Muller tube attached to a recording ratemeter according to the principle described by Sear (11). Blood flow through the tubing was approximately 15 ml. per min., measurements being made at five-minute intervals by timed collection of 6 ml. blood.

The drugs were administered in 0.85 per cent NaCl solution at an average rate of 4 ml. per min. from a pressurized flask. Flow was regulated with a needle valve and drip bottle. The average dosage of l-norepinephrine was 2.5 μgm. per Kg. per min., and of Pitressin® [2] 0.065 pressor units per Kg. per min. Administration of the drugs was started 15 minutes before injection of Rb^{86} in order to allow any transient changes in plasma potassium to subside. When Pitressin® was used the initial rise in blood pressure caused by the drug had disappeared before measurements of Rb^{86} uptake were started. The mean pulse rate was 175 per min. and the average mean blood pressure 165 mm. Hg in four dogs receiving l-norepinephrine, while in the six dogs given Pitressin® the average pulse rate was 125 per min. and the average mean blood pressure 145 mm. Hg. The hearts of three of four dogs that received l-norepinephrine showed varying degrees of intramyocardial hemorrhage, mainly subendocardial, and a decrease in the myocardial potassium concentration. The mean potassium concentration in these dogs was 66.4 mEq. per Kg. myocardium, or 20 per cent less than in the control dogs.

Precordial monitoring was performed by means of a scintillation ratemeter [3] employing a NaI crystal and a recording galvanometer with a half-time of 5 seconds. The geometry, shielding, and general arrangement of the apparatus are indicated schematically in Figure 1. The precordial radioactivity at the end of 30 minutes of Rb^{86} infusion was approximately 20 times background. Three dogs were sacrificed *in situ* by intravenous injection of 300 mg. sodium pentobarbital, and those tissues which were within the field of the counter were removed to determine what portion of the radioactivity recorded over the precordium was actually derived from the heart. This varied from 62 to 72 per cent. Almost one-half of the precordial radioactivity arising outside of the heart was derived from the anterior chest wall, while the remaining 15 to 20 per cent originated in the posterior chest wall, lungs, and the portions of the body shielded from the counter. In six dogs in which the heart was monitored separately after sacrifice, the amount of radioactivity averaged 70 per cent of the total precordial count.

The injection of Rb^{86} in 0.85 per cent NaCl solution was made into a femoral vein by means of a 50-ml. syringe driven by a variable speed motor. The usual Rb^{86} concentration of the injectant was 12 μc. per ml.,

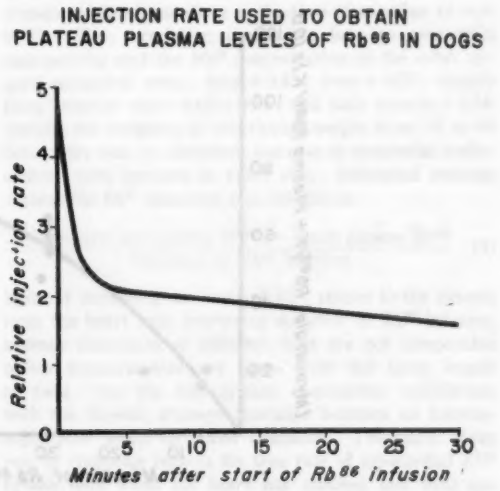

FIG. 2. THE CONTINUOUSLY DECREASING INTRAVENOUS INJECTION RATE USED TO OBTAIN NEAR CONSTANT ARTERIAL PLASMA Rb^{86} CONCENTRATION IN DOGS

[2] Supplied by Parke, Davis & Co.

[3] W. S. MacDonald Co. Type 155.

35 ml. being injected in 30 minutes. Rubidium[86] obtained as Rb_2CO_3 was neutralized with HCl and used no longer than two months after receipt in order to avoid significant contamination with long lived radio elements, such as Cs^{134}. A standard injection rate was derived by calculation from the plasma decay curve of Rb^{86} in dogs (8), with empirical modifications, Figure 2. In some cases the injection rate was altered during the procedure as required by a change in whole blood radioactivity.

Tissue specimens weighing approximately one gram were collected from several parts of the heart, from the lung, liver, and muscle of the pectoral region and spine. These samples were digested in HNO_3 for determination of radioactivity and potassium content by methods previously described (8). Specimens of arterial plasma were obtained at five-minute intervals for determination of radioactivity (12) and potassium concentration. In the initial studies plasma potassium concentration was measured using a twenty-fold dilution of plasma. However, all other plasma samples were digested with HNO_3 before dilution, and the earlier determinations were corrected to the probable values which would have been obtained with digested plasma. This correction, which amounted to a 15 per cent increase, was based upon 200 samples measured by both methods.

METHODS AND ANALYSIS OF DATA

Several assumptions were necessary to order to make calculations of myocardial potassium turnover rate and Rb^{86} clearance from the data obtained. As mentioned previously, Rb^{86} was used as if it traced potassium within the myocardium. Therefore, the mass of non-tracer material was represented by the potassium content, which was assumed not to change during the procedure. The rationale of this assumption has been given above. To extend the previous study of the relative concentrations of potassium and exchangeable rubidium in the dog's heart (8), eight animals were sacrificed three to five days after intravenous injection of Rb^{86}, and the potassium and Rb^{86} concentrations of the plasma and myocardium compared. The specific activity of exchangeable rubidium in the heart and plasma have been shown to be nearly equal after 24 hours (8), so that Rb^{86} concentration would indicate relative exchangeable rubidium content. The ratio of the Rb^{86} concentration in the myocardium to that in the plasma divided by the similar ratio for potassium averaged 1.05 ± 0.08, compared with 1.14 in four dogs reported previously. In the calculations this ratio was treated as if it were unity.

The second assumption upon which calculations were based was that the ventricular myocardium constituted a mixed homogeneous compartment exchanging at a single rate with the blood. Although this is clearly not the case, any rapidly exchanging portion containing little potassium, such as the interstitial fluid, would be undetected by the methods used. Furthermore, the results did not indicate the presence of any large slowly ex-

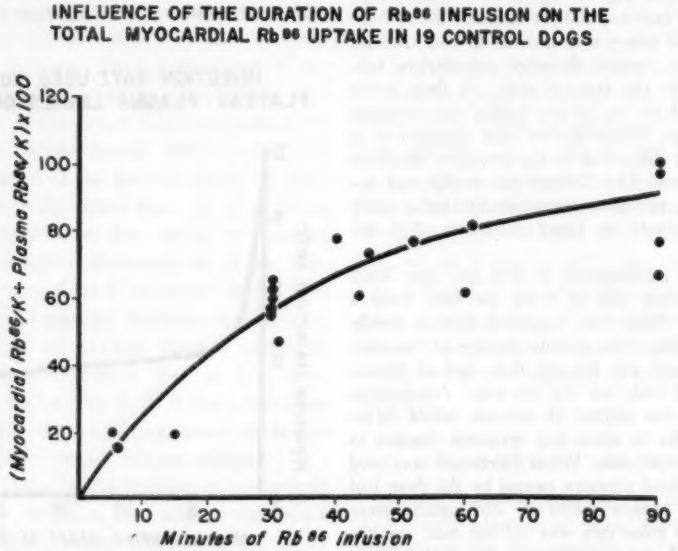

FIG. 3. THE TOTAL Rb^{86} UPTAKE IN 19 CONTROL DOGS DETERMINED AT SACRIFICE 5 TO 90 MINUTES AFTER THE START OF Rb^{86} INFUSION

The curved line represents the time course of Rb^{86} uptake in the hypothetical average dog, if the myocardium was homogeneous and exchanged Rb^{86} with the plasma at a single constant rate.

changing components. This is indicated in Figure 3 which shows the time course of the variation in individual dogs about the mean Rb^{86} uptake rate.

In making calculations from data obtained from the intact dog, it was further assumed that the precordial monitor reflected the behavior of Rb^{86} concentration in the myocardium. The actual comparison of estimates of myocardial Rb^{86} uptake made on this basis with those obtained from direct analysis of the myocardium served as the test of the usefulness of this assumption. If radioactivity over the heart reflects myocardial radioactivity, then the recording of precordial radioactivity has three attributes from which the rate of Rb^{86} uptake by the heart can be estimated. The initial rate of increase of radioactivity over the precordium is related to the initial rate at which plasma is cleared of Rb^{86} by the myocardium, while the level of precordial radioactivity at the end of the infusion of Rb^{86} is related to the maximal myocardial Rb^{86} concentration reached during the procedure and, therefore, to the average myocardial clearance of plasma Rb^{86} during the infusion. The amount of curvature of the trace of precordial radioactivity depends in part on the turnover rate of myocardial potassium. These three characteristics have been used to obtain "estimated" rates of plasma clearance and rates of turnover from the type of data obtainable in man, and these rates have been compared with their counterparts obtained by direct analysis of the heart at sacrifice. The latter have been referred to as "observed" rates of clearance and rates of turnover.

Estimated myocardial potassium turnover rate was obtained by differentiation of the time course of precordial Rb^{86} radioactivity. The continuous recording of precordial radioactivity was transcribed and the time course of its slope determined graphically. The resulting values were plotted semi-logarithmically and a single straight line drawn by inspection which appeared most nearly to represent the data. Usually there was evidence of a rapidly exchanging component during the first five minutes of the Rb^{86} infusion. This portion was ignored in choosing the predominant exchange rate, since it was considered to rise from outside the myocardium. Assuming stable potassium concentrations, the precordial radioactivity curve can be taken to represent the rise of Rb^{86}/K ratio in two compartments exchanging with plasma of constant Rb^{86}/K ratio. This may be expressed as:

$$Y_t = A(1 - e^{-at}) + B(1 - e^{-bt}) \qquad (1)$$

where

Y_t is the precordial radioactivity at any time t,

A and B are the radioactivities which would be recorded from the two compartments at complete equilibrium, *i.e.*, $t = \infty$,

b is the fraction of total non-tracer potassium entering or leaving the slowly exchanging portion per minute, and a is the similar fractional exchange rate in the fast exchanging component.

From equation (*1*)

$$\frac{dY_t}{dt} = aAe^{-at} + bBe^{-bt} \qquad (2)$$

The second term of equation (*2*), obtained graphically as previously described, was considered to represent the variation of Rb^{86}/K ratio in the myocardium.

$$b = \frac{0.69}{T\frac{1}{2}},$$

where $T\frac{1}{2}$ is the time required for the value of the second term to decrease by one-half. 100 b is equal to the per cent of the myocardial non-tracer entering or leaving the heart per minute.

Estimated initial myocardial Rb^{86} clearance. The initial rate of increase of the predominant component of the precordial radioactivity curve was obtained by evaluating the second term of equation (*2*) at $t = 0$. For each dog the resulting value was plotted against the observed initial myocardial Rb^{86} clearance rate, defined below, expressed in units of Rb^{86} cpm. per 100 Gm. myocardium per minute. The resulting empirical relationship (correlation coefficient, $r = +0.95$) was used to convert the observed initial rate of rise of precordial radioactivity in individual dogs to the units of Rb^{86} cpm. per 100 Gm. myocardium per minute. The resulting value divided by the average plasma concentration of Rb^{86} was the estimated myocardial Rb^{86} clearance, in units of ml. plasma cleared of Rb^{86} per 100 Gm. myocardium per minute.

Estimated average myocardial Rb^{86} clearance was obtained by taking advantage of the high correlation between final precordial radioactivity and the Rb^{86} concentration of the myocardium at the time of sacrifice ($r = +0.93$). The correlation coefficients between precordial radioactivity and the Rb^{86} concentration of the other organs measured were: lung $+0.85$; liver $+0.59$; muscle from anterior chest wall $+0.35$; and back muscle $+0.54$. Despite the variation in ventricular weight from 38 to 89 Gm., there was no consistent increase in precordial radioactivity with increase in heart size. Estimated average myocardial Rb^{86} clearance was defined as:

$$\frac{\text{Estimated myocardial } Rb^{86} \div \text{Mean plasma } Rb^{86}}{\text{Duration of } Rb^{86} \text{ infusion}} \qquad (3)$$

Because increasing amounts of Rb^{86} return to the plasma from the heart with increasing duration of Rb^{86} infusion, average clearances of different dogs are not comparable unless measurements are made over the same length of time. As the myocardium approaches equilibrium with the plasma, average clearance becomes an increasingly poor index of initial clearance. Therefore, mean rate of clearance reflects the true rate of myocardial Rb^{86} uptake only when the heart has attained less than approximately 40 per cent of the equilibrium Rb^{86} concentration.

Observed myocardial potassium turnover rate was calculated from the Rb^{86} and potassium concentrations of the myocardium measured at the end of the period of

Rb^{86} infusion, using the assumptions listed above. Since the myocardium was assumed to be a single compartment exchanging Rb^{86} and potassium with the plasma at a constant rate, then

$$H = C(1\text{-}e^{-bt}), \qquad (4)$$

where H is the Rb^{86}/K ratio in the myocardium at the time of sacrifice, and C is the Rb^{86}/K ratio in the heart at $t = \infty$ (C is assumed to be equal to the average Rb^{86}/K ratio observed in the plasma) and b and t are as previously defined. The values of H, C, and t were measured directly, and the turnover rate b was determined graphically.

Observed initial myocardial Rb^{86} clearance was defined as the amount of plasma at the average concentration of arterial Rb^{86} which would be required to supply the amount of Rb^{86} taken up per minute during a hypothetical moment before any Rb^{86} had begun to return from the myocardium to the plasma. This was obtained from the turnover rate and potassium concentrations as follows:

Initial myocardial Rb^{86} clearance

$$= \frac{\text{(b) (Mean myocardial K conc.)}}{\text{Mean plasma K conc.}} \qquad (5)$$

Observed mean myocardial Rb^{86} clearance was calculated in the same manner as estimated mean myocardial Rb^{86} clearance except that the myocardial Rb^{86} concentration was obtained by direct analysis of the heart muscle.

Changes in plasma Rb^{86}/K ratio occurred during Rb^{86} infusions and caused errors in the indices of Rb^{86} uptake. The influence of such variations was tested by calculating the theoretic changes produced in the time course of myocardial Rb^{86} concentration and the resulting errors in calculations of Rb^{86} uptake. Changes in plasma Rb^{86}/K ratio were assumed to be linear to simplify calculation. It is evident from Figure 4 that, in the range of exchange rates and rates of change of plasma Rb^{86}/K ratio actually encountered and investigated, a very significant error may occur in estimations of turnover rate and initial plasma Rb^{86} clearance based on differentiation of the curve of precordial radioactivity. However, little error results in the calculations of observed initial myocardial clearance of plasma Rb^{86} and turnover of potassium which depend on the relationship of final Rb^{86}/K ratio in the myocardium to the mean plasma Rb^{86}/K ratio. The error in the estimated initial clearance would be greatly reduced if the initial rather than the mean plasma Rb^{86} concentration were used in calculating the initial clearance when a definite progressive change in plasma Rb^{86}/K ratio occurred.

RESULTS

An example of the type of data obtained is given in Table I. Figure 5 illustrates the time course of precordial radioactivity in a dog receiving no drug infusion, in one receiving l-norepinephrine, and in one receiving Pitressin®. The mean plasma Rb^{86} concentration and the relationship between final precordial radioactivity and Rb^{86} concentration of the myocardium were approximately equal in these three dogs.

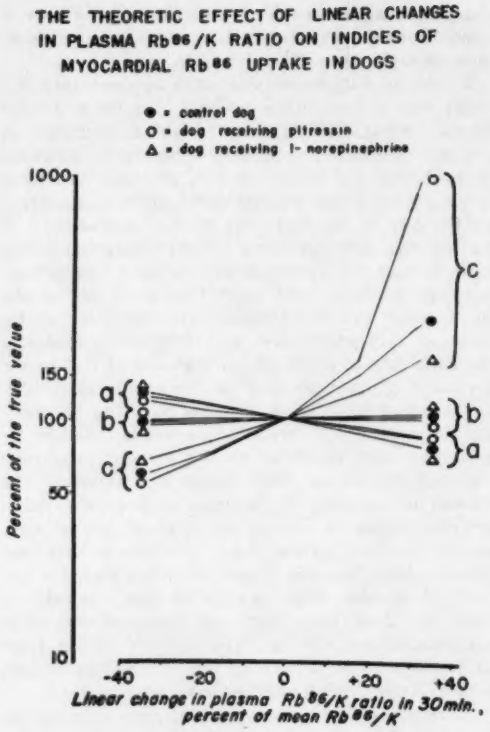

FIG. 4. THE THEORETIC EFFECTS OF LINEAR CHANGES IN PLASMA Rb^{86}/K RATIO ON THE CALCULATED INDICES OF MYOCARDIAL Rb^{86} UPTAKE IN DOGS

Since the effect depends on the actual rate of potassium turnover present, calculations were made in the range of turnover rates of myocardial potassium present in the dogs receiving Pitressin® and l-norepinephrine, as well as the control dogs. Group *a* gives the effects on the calculations of estimated initial myocardial clearance of plasma Rb^{86}, group *b* the effects on calculations of observed initial myocardial clearance of plasma Rb^{86} and turnover of potassium, and *c* the effects on calculations of estimated myocardial turnover rate of potassium.

The accuracy of estimations of turnover rate is indicated by Figure 6. Two dogs that received Rb^{86} infusions for less than 7 minutes were omitted since no record of precordial radioactivity was made, and two that were infused with Rb^{86} for 90 minutes were omitted since the myocardial

TABLE I

Data and calculations from Dog 944, wt. 14.3 Kg.

Data

	Minutes of Rb^{86} infusion					
	5	10	15	20	25	30
Rb^{86}, *cpm. per ml. plasma*	10,900	10,000	11,300	11,000	10,700	10,400
K, *mEg. per L. plasma*	3.27	3.27	3.24	3.20	3.17	3.17
Mean blood pressure, *mm. Hg*	113	112	115	118	118	119
Heart rate *per min.*	164	164	160	168	168	168

Rb^{86}, *cpm. per Gm. ventricle* (av.)	157,000
K, *mEg. per Kg. ventricle* (av.)	77.5
$T\frac{1}{2}$ from differentiation of the plot of precordial radioactivity, *min.*	54
Initial slope of principal component of curve of precordial radioactivity, converted to cpm. per Gm. ventricle per min.	9,980
Final net precordial radioactivity, converted to cpm. per Gm. ventricle	205,000

Calculations

	Observed	Estimated
K turnover of myocardium, *per cent per min.*	3.1	1.3
Initial myocardial clearance of plasma Rb^{86}, *ml. per 100 Gm. myocardium per min.*	75	93
Mean myocardial clearance of plasma Rb^{86}, *ml. per 100 Gm. myocardium per min.*	49	64

Rb^{86}/K ratio at the time of sacrifice exceeded the plasma Rb^{86}/K ratio, making it impossible to calculate the rate of turnover. Estimated turnover was consistently less than observed turnover in the dogs which did not receive drugs, averaging only one-half of the latter value. Figure 6 shows that it was not possible to predict rapid or slow rates of turnover in the control group of dogs. Abnormally low rates of turnover were apparent in two of six dogs receiving Pitressin®, since

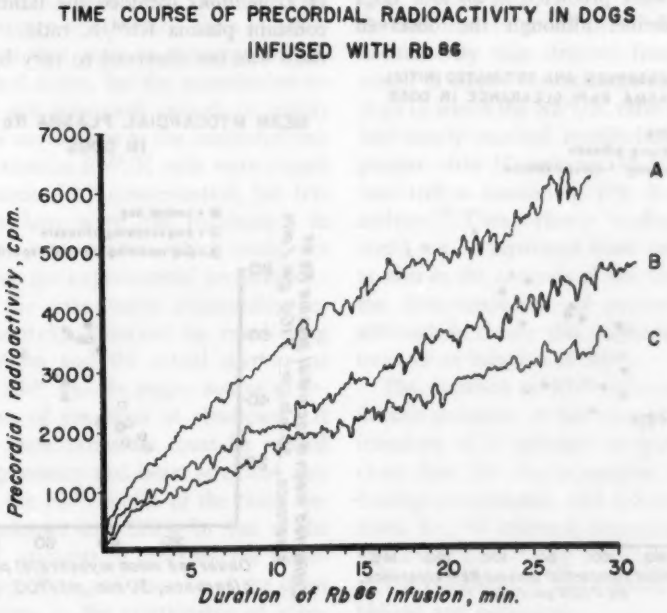

FIG. 5. THE TIME COURSE OF PRECORDIAL RADIOACTIVITY IN A CONTROL DOG (B) AND DOGS INFUSED WITH L-NOREPINEPHRINE (A) AND PITRESSIN® (C) DURING A 30-MINUTE PERIOD OF CONTINUOUS Rb^{86} ADMINISTRATION

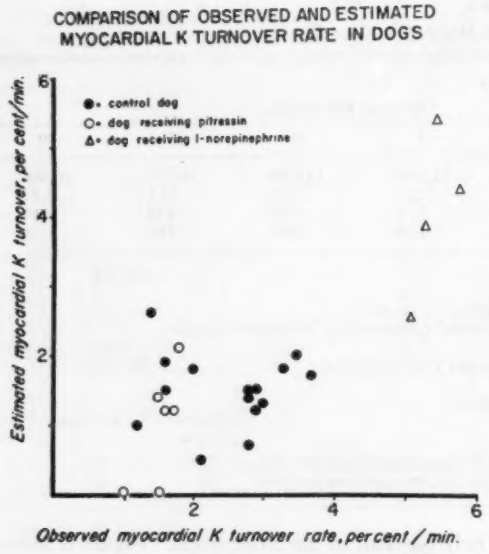
COMPARISON OF OBSERVED AND ESTIMATED MYOCARDIAL K TURNOVER RATE IN DOGS
● = control dog
○ = dog receiving pitressin
△ = dog receiving l-norepinephrine
Estimated myocardial K turnover, per cent/min.
Observed myocardial K turnover rate, per cent/min.
2
4
6

FIG. 6. COMPARISON OF OBSERVED AND ESTIMATED TURNOVER RATE OF MYOCARDIAL POTASSIUM IN DOGS

there was no detectable curvature in the tracing of precordial radioactivity. Abnormally rapid rates of turnover were predicted in all four dogs given l-norepinephrine, although the observed rates of turnover were again somewhat greater than the estimated rates.

The accuracy of estimations of the rates of initial myocardial clearances of plasma Rb^{86} is apparent from Figure 7. The standard error of estimate was 14 ml. plasma, the mean normal clearance being 70 ml. Predicted and observed clearances were necessarily equal overall because of the method of calculation.

The accuracy of estimations of mean rate of clearance in those dogs receiving Rb^{86} for 30 minutes is shown in Figure 8. The standard error of estimate was 10 ml. plasma, the mean control value being 50 ml. There was no consistent difference between the values for estimated and observed mean clearances because an experimentally derived factor was used to obtain the estimated myocardial Rb^{86} concentration from the final level of precordial radiation. In accordance with considerations discussed previously, decreased uptake caused by Pitressin® was detected, whereas the small increase in mean clearance occurring in dogs receiving l-norepinephrine was not.

Factors affecting the accuracy of the three types of estimations included the failure to maintain a constant plasma Rb^{86}/K ratio. In 18 dogs this ratio was not observed to vary by as much as 10

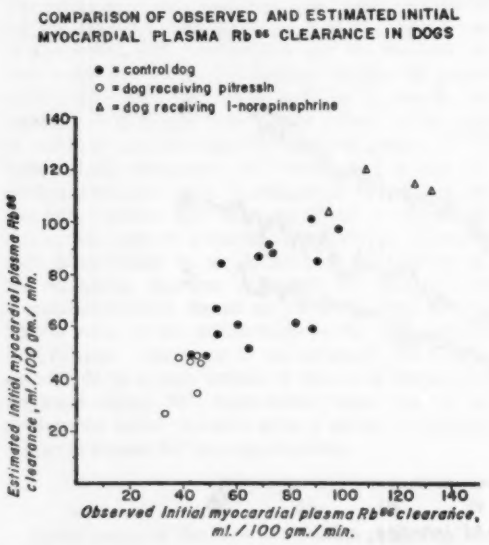
COMPARISON OF OBSERVED AND ESTIMATED INITIAL MYOCARDIAL PLASMA Rb 86 CLEARANCE IN DOGS
● = control dog
○ = dog receiving pitressin
△ = dog receiving l-norepinephrine
Estimated initial myocardial plasma Rb 86 clearance, ml./100 gm./min.
Observed initial myocardial plasma Rb 86 clearance, ml./100 gm./min.
20
40
60
80
100
120
140

FIG. 7. COMPARISON OF THE VALUES OF OBSERVED AND ESTIMATED INITIAL MYOCARDIAL PLASMA Rb^{86} CLEARANCE IN DOGS

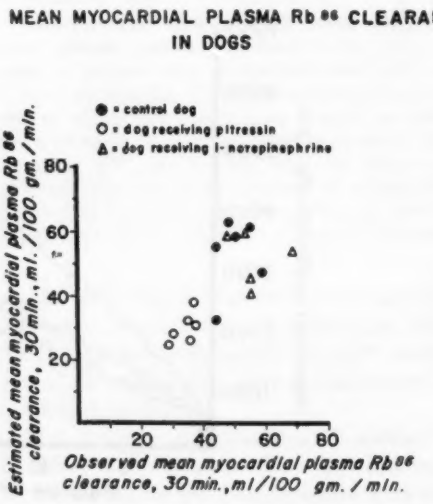
MEAN MYOCARDIAL PLASMA Rb 86 CLEARANCE IN DOGS
● = control dog
○ = dog receiving pitressin
△ = dog receiving l-norepinephrine
Estimated mean myocardial plasma Rb 86 clearance, 30 min., ml./100 gm./min.
Observed mean myocardial plasma Rb 86 clearance, 30 min., ml/100 gm./min.
20
40
60
80

FIG. 8. COMPARISON OF THE VALUES OF OBSERVED AND ESTIMATED MEAN MYOCARDIAL PLASMA Rb^{86} CLEARANCE FOR A 30-MINUTE PERIOD IN THE DOG

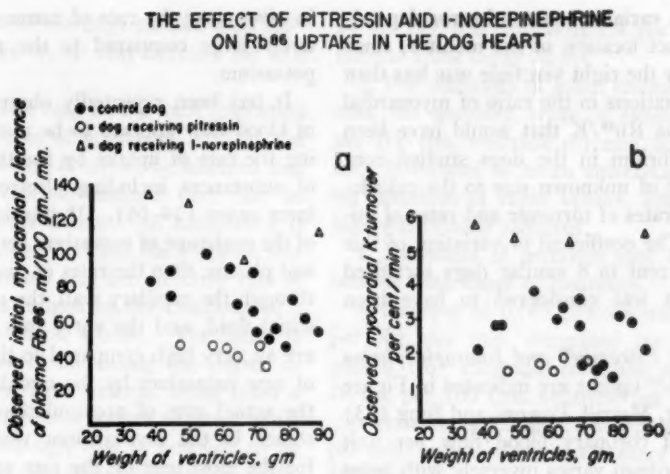
THE EFFECT OF PITRESSIN AND I-NOREPINEPHRINE
ON Rb86 UPTAKE IN THE DOG HEART
● = control dog
○ = dog receiving pitressin
Δ = dog receiving l-norepinephrine
a
b
Observed initial myocardial clearance of plasma Rb86, ml/100 gm./min.
Observed myocardial K turnover per cent/min
Weight of ventricles, gm.
Weight of ventricles, gm.

FIG. 9. THE EFFECT OF PITRESSIN® AND L-NOREPINEPHRINE ON THE OBSERVED INITIAL MYOCARDIAL CLEARANCE OF PLASMA Rb^{86} (a) AND THE OBSERVED MYOCARDIAL POTASSIUM TURNOVER RATE (b) IN DOGS

per cent of the mean during the Rb^{86} infusion, while in the remaining 9 dogs the Rb^{86}/K ratio declined as much as 22 per cent or increased as much as 33 per cent of the mean value. In general these changes in plasma Rb^{86}/K ratio were associated with errors in estimated rates of clearance and turnover that were in the expected directions as outlined above, but the quantitative relationships were not consistent enough to justify the application of corrections to the estimated values. Some variations in Rb^{86}/K ratio were caused by changes in plasma Rb^{86} concentration, but frequently the variations were due to changes in plasma potassium concentration which could not be detected during the experimental procedure.

Variations in the quantitative relationship between the radioactivity observed by monitoring over the precordium and the actual myocardial concentration of Rb^{86} was the major source of error in estimations of the rates of clearance. It is apparent that such variations must be related to variations in geometry and heart size, and also to differences in the Rb^{86} uptake of the other tissues under the monitor in relation to that of the heart. However, attempts to correlate the radioactivity of the lung, liver, and muscles of the chest wall with variations in the relationship of myocardial Rb^{86} concentration and precordial radioactivity were unsuccessful. The presence of important amounts of slowly exchanging tissue under the precordial monitor would result in estimated rates of turnover which would be consistently lower than observed rates, as was the case. Dissection of the animals after sacrifice showed that 10 to 20 per cent of the precordial radioactivity was derived from skeletal muscle, which is known to exchange slowly (8). In two dogs in which the Rb^{86}/K ratio in the myocardium had nearly reached equilibrium with that of the plasma after 90 minutes of Rb^{86} infusion, there was still a continuing rise in precordial radioactivity. These slowly exchanging components could not be separated from the myocardial component in the analysis of the time course curve of the first derivative of precordial radioactivity, although in theory this might be possible after 90 minutes of infusion of Rb^{86}.

The duration of Rb^{86} infusion appeared to influence estimates of the rates of Rb^{86} uptake since infusions of 15 minutes or less provided insufficient data for the separation of rapidly equilibrating components, and infusions of long durations, *i.e.*, 90 minutes, accentuated errors caused by slowly exchanging components and variations in the comparative distribution of non-tracer rubidium and potassium.

The assumption that the right and left ventricles had the same geometrical relationship to the moni-

tor represented a variable source of error depending upon the exact location of the monitor, since uptake of Rb^{86} by the right ventricle was less than by the left. Variations in the ratio of myocardial Rb^{86}/K to plasma Rb^{86}/K that would have been present at equilibrium in the dogs studied contributed an error of unknown size to the calculation of observed rates of turnover and rates of initial clearance. The coefficient of variation of this ratio was 8 per cent in 8 similar dogs sacrificed after equilibrium was considered to have been achieved.

The effects of Pitressin® and l-norepinephrine on the rate of Rb^{86} uptake are indicated in Figure 9. Since Spencer, Merrill, Powers, and Bing (13) have shown that coronary blood flow per unit weight of myocardium varies inversely with heart size, the indices of Rb^{86} uptake have been plotted with the values for ventricular weight. The differences in mean heart rate among the three groups of dogs were relatively small, as noted previously, and there was no definite relationship between heart rate and rate of uptake of Rb^{86} within the individual groups.

DISCUSSION

It is evident that differentiation of the time course of precordial radioactivity did not result in even a gross indication of the rate of uptake of Rb^{86} by the myocardium. This method probably is more suited for conditions in which the rate of turnover is greater than that encountered in these dogs, since with greater curvature of the plot of radioactivity the graphic methods used for analysis would be expected to be more reliable and interference by slowly exchanging components less important. On the other hand, estimations of initial rate of clearance of plasma Rb^{86} by the myocardium were reasonably reliable for the detection of both increased and decreased rates of uptake. Estimations of the mean rate of Rb^{86} clearance appeared to be reliable only for the detection of diminished Rb^{86} uptake. It should be noted that if reproducible and reliable estimates of the rate of turnover of potassium by the myocardium could be combined with determinations of the rate of initial myocardial clearance of plasma potassium it would be possible to detect lowered concentration of potassium in the myocardium *in vivo*, since the rate of turnover would be relatively large compared to the rate of uptake of potassium.

It has been repeatedly observed that the rate of blood flow appears to be a major factor limiting the rate of uptake by the tissues of a variety of substances including electrolytes, water, and inert gases (14–16). If this is true in the case of the exchange of potassium between myocardium and plasma, then the rates of passage of potassium through the capillary wall, the mixing with interstitial fluid, and the entry into myocardial fibers are all very high compared to the rate of delivery of new potassium by the circulation. Therefore, the actual rate of accumulation of a tracer substance in the myocardium would be dependent for the most part on the rate at which the tracer was brought to the heart by the coronary blood. H. L. Conn and J. S. Robertson (17) have stated that in a series of dogs the amount of K^{42} entering the interstitial fluid of heart muscle from the plasma was approximately equal to the total amount delivered by the coronary blood, assuming that the data of others (18) for the coronary blood flow in dogs were applicable to the animals used in their experiments. In the studies reported here, it was found that drugs which are known to affect coronary blood flow affect the rate of uptake of Rb^{86} by the myocardium as a whole in the manner which would be expected if Rb^{86} uptake were dependent on the rate of coronary blood flow. The observed myocardial potassium turnover rate estimated with Rb^{86} in these dogs was correlated with the concentration of potassium in the plasma ($r = +0.67$), whereas the observed initial myocardial clearance of plasma Rb^{86} showed no significant correlation with the concentration of potassium in the plasma ($r = +0.24$, σ of $r = 0.25$). This is consistent with the uptake by the myocardium of all, or a constant fraction, of the tracer in coronary arterial blood. These data do not exclude the possibility that these drugs produced their effects through alteration in cellular metabolic or chemical factors. Conn and Robertson (17) have found the $T_{1/2}$ value of the slowly exchanging component of myocardial potassium in the dog to average 45 minutes; whereas, in the normal dogs reported here this value was 25 minutes. The smaller size, and subsequently relatively higher rates, of

coronary blood flow of the dogs studied with Rb^{86} is one of the factors which may account for this difference.

Although blood flow may be the most important factor limiting the rate of uptake of Rb^{86} by the heart of the normal dog, it cannot be inferred that capillary or extravascular factors may not be of significance in heart disease. In addition, it is necessary to distinguish between the absolute rate of blood flow to a tissue and the effective rate of blood flow, which is judged by the clearance of a substance from the blood stream. The heart is known to be capable of increasing the degree of extraction of oxygen from arterial blood from a normal value of 70 per cent to one as great as 91 per cent (19). Although this is believed to be caused by a change in the oxygen diffusion gradients, it is possible that local circulatory readjustments such as the closing of arteriovenous shunts (20) or the re-arrangement of capillary circulation might play a role in determining the efficiency of extraction of oxygen or other substances such as Rb^{86} from flowing blood. Johnson, Cavert, and Lifson (15) have shown that in the isolated perfused heart of the dog the initial degree of extraction of D_2O from coronary blood was 100 per cent, and Conn and Robertson (17) considered K^{42} extraction by the dog heart *in situ* to be 100 per cent. However, these workers found that after less than one minute the extraction of arterial K^{42} had fallen to approximately 65 per cent. This latter figure is essentially in agreement with the experience with Rb^{86} in six dogs submitted to thoracotomy and cannulation of the coronary sinus in this laboratory. The apparent initial extraction of Rb^{86} from arterial plasma averaged 70 per cent in these dogs, although the existence of an early rapidly exchanging component of myocardial potassium would not have been detected by the methods used.

SUMMARY

1. Methods suitable for estimating the rate of myocardial uptake of Rb^{86} in man were tested in 27 dogs because of the probable relationship of Rb^{86} uptake to coronary blood flow and to possible extravascular factors affecting the exchange of metabolites.

2. Estimates of initial myocardial clearance of plasma Rb^{86} had a standard error of estimate of 14 ml. per 100 Gm. ventricle per min., the mean normal value being 70 ml.

3. For estimates of mean clearance for 30 minutes, the standard error of estimate was 10 ml. per 100 Gm. ventricle per min. and the mean 50 ml. Estimates of mean clearance were unsuited for detection of rapid uptake, although slow uptake was reliably reflected.

4. Estimates of turnover rate were much less reliable than those of clearance.

5. Infusion of 0.065 pressor unit of Pitressin® per Kg. per min. decreased the average initial plasma Rb^{86} clearance from the normal value of 70 ml. per 100 Gm. ventricle per min. to 42 ml.

6. Infusion of 2.5 μgm. l-norepinephrine per Kg. per min. increased initial clearance to 116 ml. per 100 Gm.

7. These changes in plasma Rb^{86} clearance were in the direction expected from the known effects of these drugs on coronary blood flow. The ability to detect such changes with a reasonable degree of accuracy in dogs by external monitoring over the heart supports a trial of these procedures in man.

ACKNOWLEDGMENT

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DIFFERENCES IN THE RATE OF Rb^{86} UPTAKE BY SEVERAL REGIONS OF THE MYOCARDIUM OF CONTROL DOGS AND DOGS RECEIVING L-NOREPINEPHRINE OR PITRESSIN®[1]

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The rate at which Rb^{86} enters the myocardium from the blood is determined by the rate of coronary blood flow and by the kinetics of potassium within the myocardium. In preparation for a study of the effects of disease on the rate of myocardial Rb^{86} uptake in man, a trial of methods was made in a series of dogs. During this study the rate of Rb^{86} uptake in several regions of the heart of control dogs was measured, and the effects of l-norepinephrine and Pitressin® on differences in regional Rb^{86} uptake were determined, since these drugs respectively increase and decrease overall coronary blood flow (1, 2).

MATERIALS AND METHODS

Details of the materials and methods used have been described elsewhere (3). These data on Rb^{86} uptake of different regions of the heart were obtained from the same dogs in which precordial monitoring was carried out.

Mongrel dogs weighing 6.7 to 14.3 Kg. were anesthetized with sodium pentobarbital and infused intravenously with Rb^{86} at a continuously decreasing rate for periods of 5 to 90 minutes in order to attain nearly constant levels of Rb^{86} in the plasma of arterial blood. The radioactivity of whole blood was monitored in a small external arterio-venous shunt from which samples were taken at 5-minute intervals for determination of plasma Rb^{86} and potassium concentrations. Frequent recordings of heart rate and mean arterial blood pressure were made after 100 to 150 mg. heparin had been given. Four dogs received a mean dosage of 2.5 μgm. per Kg. per min. l-norepinephrine intravenously for 30 minutes and six dogs received a mean of 0.065 pressor units per Kg. per min. Pitressin® for a similar period, during which time they also received Rb^{86}. The dogs were sacrificed by rapidly opening the chest and removing the heart. Specimens were obtained from several regions of the heart, from the lung and liver, and from the skeletal muscle of the pectoral region and spine.

Specimens obtained from the hearts of 29 dogs were as follows: full thickness of the left ventricle in the apical region, full thickness of the left ventricle in the basilar region, full thickness of the mid-portion of the interventricular septum, a portion of the thickest part of the left ventricle divided approximately into outer, middle, and inner thirds, a similar specimen from the right ventricle divided into inner and outer halves, and specimens of the full thickness of the right ventricle and from both auricles. The latter included the appendages and a portion of the adjacent wall of the auricle. The potassium and Rb^{86} concentrations of all specimens were determined after digestion in HNO_3.

METHODS OF ANALYSIS

The rationale of the methods of analysis and the details of the procedure have been presented elsewhere (3). It was assumed that Rb^{86} traced potassium in the myocardium, that the individual portions of the myocardium could be considered homogeneous compartments

TABLE I

Mean potassium concentration of various regions of the hearts of 19 control dogs, and the mean ratio of myocardial Rb^{86}/K to plasma Rb^{86}/K of the same regions of the hearts of eight dogs sacrificed more than 72 hours after Rb^{86} injection

Region of the myocardium	(Myocardial Rb^{86}/K ÷ Plasma Rb^{86}/K) × 100, at equilibrium	K. conc. *mEq. per Kg.*
Mean of six specimens from the left ventricle	106.1 ± 7.6	82.2 ± 4.5
Outer third of left ventricle	108.0	83.3
Middle third of left ventricle	105.8	84.0
Inner third of left ventricle	104.0	80.3
Mean of three specimens from the right ventricle	103.3	81.6
Outer half of right ventricle	104.5	82.2
Inner half of right ventricle	102.9	80.9
Right atrium	115.6	59.5
Left atrium	114.1	67.2

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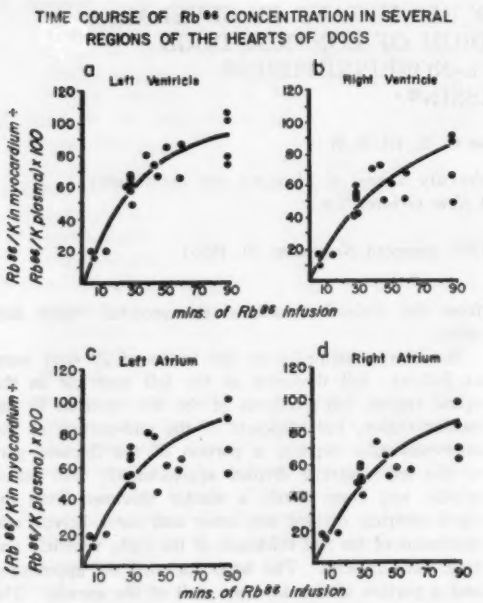
TIME COURSE OF Rb86 CONCENTRATION IN SEVERAL REGIONS OF THE HEARTS OF DOGS
a Left Ventricle
b Right Ventricle
c Left Atrium
d Right Atrium
(Rb86/K in myocardium ÷ Rb86/K plasma) x 100
mins. of Rb86 infusion

FIG. 1. THE TOTAL Rb^{86} UPTAKE IN THE WALLS OF EACH CHAMBER OF THE HEARTS OF 19 CONTROL DOGS AFTER 5 TO 90 MINUTES OF Rb^{86} INFUSION

The lines indicate the average uptake at any time. This was obtained from the mean of all uptake rates without regard to duration of infusion of Rb^{86}.

which exchanged with plasma at constant rates, and that potassium concentrations did not change during the time that the measurements were being made. A portion of the data which support these assumptions is given in Table I and Figure 1. Rb^{86} uptake has been expressed in terms of the clearance of Rb^{86} by the myocardium and also in terms of the apparent turnover rate of potassium between the myocardium and the circulating plasma, which is defined as the fraction of the myocardial potassium which exchanges with the plasma in one minute. Potassium turnover rates were of interest because the potassium concentration of various regions of the myocardium differed, and it was felt that under some circumstances the potassium turnover rate might reflect the amount of effective plasma flow per unit amount of contractile tissue more accurately than would a simple clearance measurement, which is expressed as uptake per Gm. of tissue.

The turnover rate of potassium within the myocardium was obtained graphically from the following relationship.

$$H = C\ (1\text{-}e^{-bt})$$

where

H is the Rb^{86}/K ratio in the myocardium at the time of sacrifice,

C is the Rb^{86}/K ratio of the heart at $t = \infty$ (C is assumed to be equal to the average Rb^{86}/K ratio observed in the plasma.),

t is the duration of Rb^{86} infusion in minutes, and

b is the fraction of total myocardial potassium entering or leaving per minute, *i.e.*, the turnover rate.

If the blood flow is slow in comparison to the flux of potassium into and out of the myocardial fiber, then calculations by this method give a value for apparent potassium turnover which is lower than the true rate of flux.

The initial clearance of plasma Rb^{86} by the myocardium was defined as the amount of arterial plasma which would be required to supply the Rb^{86} taken up during a hypothetical moment before any Rb^{86} had begun to return from the myocardium to the plasma. It was therefore a minimal value for rate of plasma flow.

$$\text{Initial myocardial } Rb^{86} \text{ clearance} = \frac{\text{(b) (Myocardial K conc.)}}{\text{Mean plasma K conc.}}$$

Rb^{86} uptake by organs other than the heart was expressed as clearance of plasma Rb^{86} in ml. per Gm. per 30 min. which is equal to

$$\text{Organ } Rb^{86} \text{ cpm. per Gm.} \div \text{Plasma } Rb^{86} \text{ cpm. per ml.}$$

in dogs given Rb^{86} for a 30-min. period.

No correction has been made in the calculations for differences in the equilibrium ratio of myocardial Rb^{86}/K divided by plasma Rb^{86}/K in the various regions of the heart. These values for 8 dogs are given in Table I.

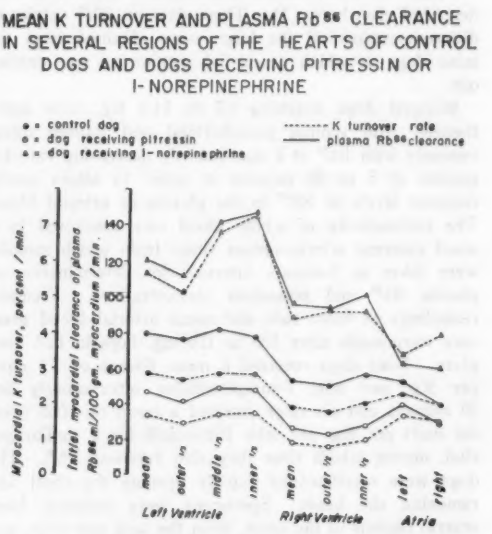

FIG. 2. MEAN POTASSIUM TURNOVER AND CLEARANCE OF PLASMA Rb^{86} IN SEVERAL REGIONS OF THE HEARTS OF CONTROL DOGS AND DOGS RECEIVING PITRESSIN® OR L-NOREPINEPHRINE

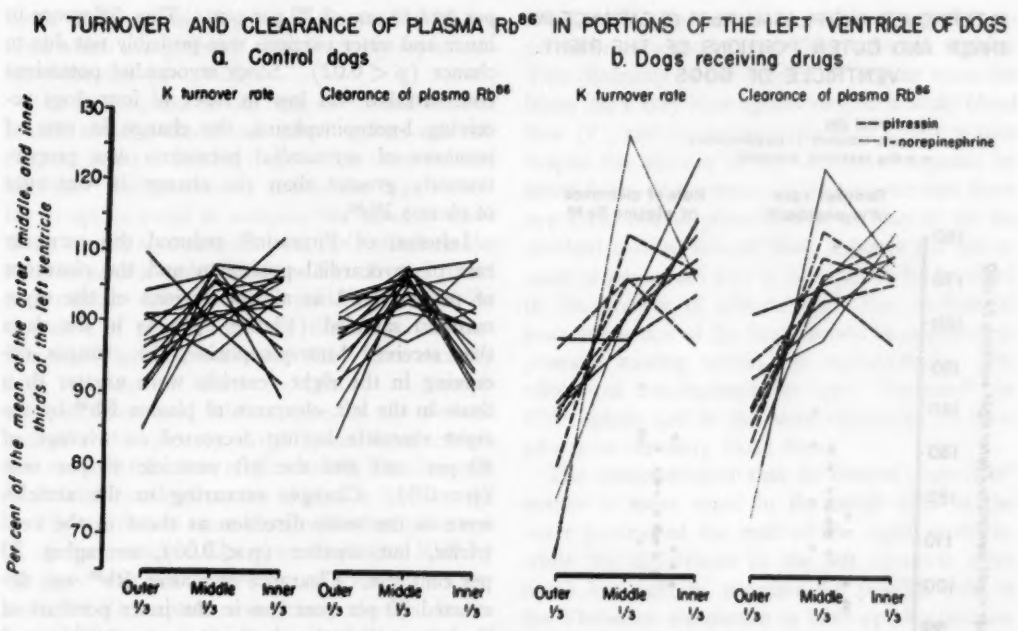

FIG. 3. POTASSIUM TURNOVER AND CLEARANCE OF PLASMA Rb^{86} IN THE OUTER, MIDDLE AND INNER THIRDS OF THE WALL OF THE LEFT VENTRICLE IN CONTROL DOGS (a) AND DOGS RECEIVING INFUSIONS OF L-NOREPINEPHRINE OR PITRESSIN® (b)

RESULTS

The mean values of initial clearance of plasma Rb^{86} and turnover rate of potassium for several regions of the myocardium in control dogs and those receiving Pitressin® or l-norepinephrine are indicated in Figures 2, 3, and 4. Since no significant differences were noted in the results obtained in specimens from the apex of the left ventricle, base of the left ventricle, and from the interventricular septum, these values have not been recorded separately. The statistical significance [2] of some of the differences in Rb^{86} uptake of various regions of the heart is indicated below by the appropriate p value.

COMMENT

Figure 2 indicates that there was a greater clearance of plasma by the left ventricle of control dogs than by the right ventricle ($p < 0.01$). The clearance of plasma Rb^{86} and the turnover rate of potassium in the left ventricle averaged 45 per cent more than in the right, the potassium concentrations of the two being nearly equal (Table I). Clearance of plasma Rb^{86} by the auricles was only slightly lower than by the right ventricle, while the turnover rate of potassium was higher in the atria than in the right ventricle, in association with the relatively low potassium concentration in the atria. Clearance of Rb^{86} ($p < 0.01$) and turnover of potassium ($p = 0.07$) were less in the portion of the right atrium sampled than in the left atrium.

In Figure 3 the small differences in the Rb^{86} uptake by the outer, middle, and inner portions of the left ventricle are apparent. Clearance of plasma Rb^{86} by the middle portion of the wall of the left ventricle averaged 8.0 per cent more than by the outer third ($p = 0.01$) and 5.8 per cent more than by the inner third ($p < 0.01$). Clearance was 2.0 per cent greater by the inner portion than by the outer portion, but this may have been due to chance ($p > 0.4$).

The relationship of the values of turnover of

[2] The Fisher test for unique samples was used where applicable.

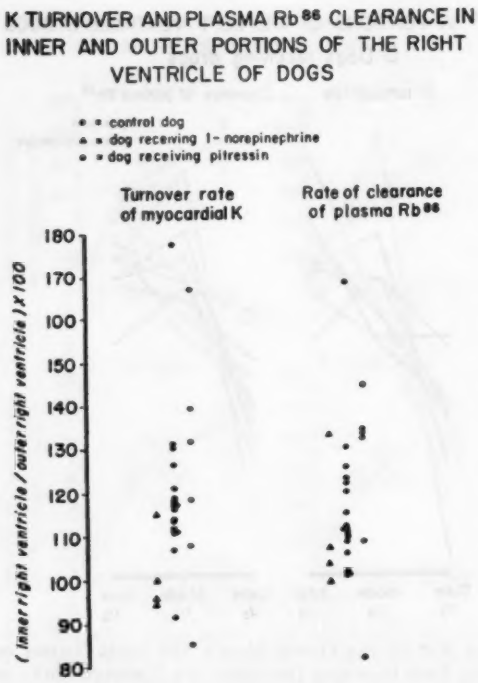

FIG. 4. THE POTASSIUM TURNOVER AND CLEARANCE OF PLASMA Rb^{86} IN THE INNER AND OUTER PORTIONS OF THE RIGHT VENTRICLE OF CONTROL DOGS AND DOGS RECEIVING L-NOREPINEPHRINE OR PITRESSIN®

myocardial potassium were similar, except that the middle and inner portions were nearly the same, and turnover was 6.2 per cent greater in the inner portion than in the outer ($p < 0.05$).

The clearance of plasma Rb^{86} and the turnover of myocardial potassium in the right ventricle were higher in the inner than in the outer portion ($p < 0.01$), the difference averaging 15 per cent (Figure 4).

Clearance of plasma Rb^{86} and turnover of myocardial potassium were increased in all regions of the myocardium by the infusion of l-norepinephrine (Figure 2). The rate of Rb^{86} uptake was increased more in the ventricles than in the atria ($p < 0.02$), and more in the right ventricle (77 per cent) than in the left (50 per cent) ($p = 0.2$). In the left ventricle the increase in clearance was greater in the inner portion than in the outer portion ($p < 0.02$). This accentuated the difference between the clearance in inner and outer portions noted in the controls from a value of 2 per cent to one of 29 per cent. This difference in inner and outer portions was probably not due to chance ($p < 0.02$). Since myocardial potassium concentration was low in three of four dogs receiving l-norepinephrine, the change in rate of turnover of myocardial potassium was proportionately greater than the change in clearance of plasma Rb^{86}.

Infusion of Pitressin® reduced the turnover rate of myocardial potassium and the clearance of plasma Rb^{86} in all the regions of the myocardium sampled (Figure 2). As in the dogs that received l-norepinephrine, the changes occurring in the right ventricle were greater than those in the left, clearance of plasma Rb^{86} by the right ventricle having decreased an average of 49 per cent and the left ventricle 40 per cent ($p = 0.3$). Changes occurring in the auricles were in the same direction as those in the ventricles, but smaller ($p < 0.06$), averaging 20 per cent less. Clearance of plasma Rb^{86} was decreased 20 per cent less in the inner portions of the left ventricle than in the outer ($p < 0.03$), and the 2.0 per cent difference between these regions

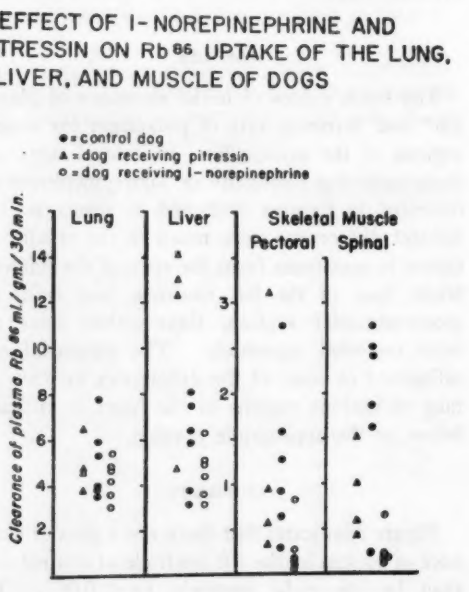

FIG. 5. CLEARANCE OF PLASMA Rb^{86} BY THE LUNGS, LIVER, AND SKELETAL MUSCLE FROM THE PECTORAL AND SPINAL REGIONS OF CONTROL DOGS AND DOGS WHICH RECEIVED INFUSIONS OF L-NOREPINEPHRINE OR PITRESSIN®

noted in the normal dogs was increased to 17 per cent, which was probably a qualitatively reproducible difference ($p < 0.02$).

The Rb^{86} uptake of the lung, liver, and skeletal muscle of the pectoral and spinal regions was calculated in the 6 control dogs which were infused with Rb^{86} for 30 minutes and in the dogs receiving drugs, in order to compare the effect of these drugs on the myocardium with their effect on the other organs sampled (Figure 5). It was found that neither drug had any noticeable effect on clearance of plasma Rb^{86} by the lung, but that Pitressin® greatly decreased Rb^{86} clearance by both the muscles tested ($p < 0.01$). l-Norepinephrine decreased Rb^{86} uptake by the skeletal muscle of the spine ($p < 0.05$), but its effect was less marked than that of Pitressin®. The decrease in skeletal muscle clearance of plasma Rb^{86} caused by Pitressin® was more than twice that produced in cardiac muscle. Clearance of plasma Rb^{86} by the liver was not apparently affected by Pitressin®, but Rb^{86} uptake was greatly elevated in the livers of three of the four dogs that received l-norepinephrine.

GENERAL DISCUSSION

These variations in Rb^{86} uptake rate reflect differences in the rate at which Rb^{86} is brought to the area by the blood, or the actions of unknown factors affecting the flux of rubidium across the walls of the capillary and myocardial fiber or affecting the mixing of rubidium within the interstitial fluid and cell. A variety of evidence favors the view that the flux of potassium and Rb^{86} across cellular membranes in the heart is rapid by comparison with the rate at which these substances can be delivered by the coronary blood, and that therefore the rate of coronary blood flow is ordinarily the factor most likely to limit Rb^{86} uptake (3). In the case of organs other than the heart, it has been found that blood flow is a major factor limiting the rate of uptake of water and inert gases, as well as electrolytes (4–6). Rb^{86} is taken up rapidly by the vascular thyroid and adrenal glands (7), even though these organs are not known to have unusually high cellular exchange rates of potassium or rubidium. The effects of Pitressin® and l-norepinephrine on Rb^{86} uptake of resting muscle in the dogs reported here are in the directions which would be expected if regional blood flow affected uptake. This obviously is not true of all organs since the brain has a very slow uptake despite a rapid blood flow (7), and the erythrocytes have a slow uptake despite the absence of any limitation imposed by blood flow. It is reasonable to suppose that there is a rapid flux of potassium in and out of the individual cardiac muscle fiber, because the movement of potassium ions is believed to be involved in the process of contraction. The mechanical kneading action of the heart would be expected to promote mixing within the myocardium. The effects of l-norepinephrine and Pitressin® on Rb^{86} uptake are in the same directions as their effects on coronary blood flow.

The demonstration that in control dogs Rb^{86} uptake is more rapid in the inner than in the outer portion of the wall of the right ventricle, while the differences in the left ventricle were much less definite, suggests the possible role of the Thebesian circulation in Rb^{86} uptake, because these vessels are known to be more numerous in the right ventricle of the dog than the left (8). Quantitative variations in the effects of Pitressin® and l-norepinephrine on different regions of the heart may indicate variations in local blood flow caused by the humoral control of blood vessels within the myocardium.

SUMMARY

1. When control dogs were given Rb^{86} intravenously, the mean rate of Rb^{86} uptake by the left ventricle was 45 per cent faster than by the right ventricle.

2. The rate of Rb^{86} uptake in the atria was similar to that in the right ventricle.

3. The inner portion of the right ventricle consistently took up Rb^{86} more rapidly than did the outer portion.

4. In dogs receiving l-norepinephrine or Pitressin®, the uptake was significantly more rapid in the inner portion of the left ventricle than in the outer portion.

5. Pitressin® and l-norepinephrine produced changes in Rb^{86} uptake rate which were in the same direction as their known effects on the rate of coronary blood flow.

6. These differences in the rate of Rb^{86} uptake are believed to be related to differences in the rate of effective blood flow, although other factors have not been eliminated.

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STUART CLOTTING DEFECT. I. SEGREGATION OF AN HEREDITARY HEMORRHAGIC STATE FROM THE HETEROGENEOUS GROUP HERETOFORE CALLED "STABLE FACTOR" (SPCA, PROCONVERTIN, FACTOR VII) DEFICIENCY

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In 1951, Alexander, Goldstein, Landwehr, and Cook (1) described a patient with a congenital hemorrhagic diathesis of an unusual type. There was a normal prothrombin concentration, but a prolonged prothrombin time which could be corrected by serum and serum fractions but not by $BaSO_4$-adsorbed plasma or serum. The clotting factor deficient in the plasma of this patient differed, therefore, from prothrombin, Factor V, accelerator globulin (Ac-G), and antihemophilic factor (AHF). The normal analogue of the factor deficient in this patient was relatively heat stable, adsorbable by barium sulphate and diminished in dicoumarol plasma. The patient was believed to lack the precursor of SPCA (serum prothrombin conversion accelerator) a factor which had been studied previously (2).

Independently in 1951, Koller, Loeliger, and Duckert (3) and Owren (4) recognized the presence of clotting factors designated Factor VII and proconvertin, respectively. These two factors were believed to be identical with each other and with SPCA. Although the three terms have been used synonymously since that time, the literature shows no evidence that the identity has been rigorously established. Since the case of Alexander, Goldstein, Landwehr, and Cook (1), 29 other cases (Table I) of a congenital deficiency of these presumably identical factors have been described (5–24). Our present studies indicate that the patient of Crockett, Shotton, Craddock, and Leavell (25) also belongs in the same group. The thromboplastin generation test has been performed on 16 of the 31 patients. It has been normal in six, abnormal in nine, and questionable in one (see Table I). This suggests that the cases diagnosed as Factor VII deficiency, SPCA deficiency and hypoproconvertinemia may be a heterogeneous group.

The following paper describes a follow-up study of one of the cases (R. S.) previously studied by Lewis, Fresh, and Ferguson (9). It will be shown that the factor deficient in this patient is similar to but not identical with the one lacking in the patient of Alexander (1), and is identical with the factor Crockett's patient lacks (25). These findings establish the heterogeneity of the group of patients listed in Table I. The factor our patient lacks will be referred to hereafter as the Stuart factor after the patient's surname. The present communication describes the properties of the Stuart factor and its role in blood coagulation. The mode of inheritance and certain other genetic considerations will be reported in a separate communication (26).

MATERIALS

Imidazole buffer, pH 7.3, was prepared according to Mertz and Owen (27). *Factor V* was prepared from plasma by the technique of Owren (28). *Prothrombin* was prepared from human plasma by the method of Biggs and Macfarlane (29). The *Russell's viper venom* ("Stypven") used in the experiments of Table III was the Burroughs Wellcome product in a 1:10,000 concentration, higher concentrations being inhibitory. In other experiments a 1:20,000 dilution was also used. *Antihemophilic factor (AHF)* was a fraction of bovine plasma rich in AHF prepared by the method of Bidwell (30). It was used in a concentration of 100 mg. per 100 ml.

METHODS

Blood was collected, and *plasma* was prepared by methods previously described by Graham, McLendon, and Brinkhous (31). *$Al(OH)_3$ adsorption* was performed by the method of Biggs and Macfarlane (29). Serum factors adsorbed by this technique were eluted with a phosphate buffer at pH 8. Clotting time was performed by the method of Lee and White (32). *Pro-*

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TABLE I

Summary of cases in literature

Case	Authors	Year	Sex	Clinical severity	Prothrombin time (sec.) Patient	Prothrombin time (sec.) Control	Thromboplastin generation test	Effect of "stypven" on clotting time
1	Crockett and associates (25)	1949	F	Severe	55	14.2	Abnormal*	Not corrective*
2	Alexander and associates (1)	1951	F	Severe	72	17	Normal (43)	
3	Beaumont and Bernard (5)	1953	M	Severe	65	13		
4	Owren (6)	1953	M	Severe	55–60	14		Corrective (39)
5			F	Severe	1–3% proconvertin			
6	Hagen and Watson (7) and Frick and Hagen (8)	1948 1953	F	Severe	52.5	12		
7	Lewis and associates (9)	1953	F	Severe	73–110			
8			M	Severe	48–55		Abnormal*	Not corrective*
9	Marciniakowna and associates (10)	1953	F	Severe	153	14–19		
10	Wurzel and associates (11)	1954	F	Mild	17	12–13		
11	Jenkins (12)	1954	M	Severe	39–70	15–17	Normal†	Corrective (38)
12	Quick and associates (13)	1955	F	Severe	65–70	12		
13			F	Severe	26–45	12		
14			F	Mild	14	12		
15	Long and associates (14)	1955	F	Severe	120	18		
16			F	Severe	160	18		
17	de Vries and associates (15)	1955	M	Severe	20	15.5	Abnormal	
18			M	Mild	17	12.8	Abnormal	
19			M	Mild	19.8	14.7	Abnormal	
20	Hicks (16)	1955	F	Severe	28–105	11.5–14	Normal	Corrective
21	Hule and associates (17)	1955	M	Severe	200	13–14		
22	Koch and associates (18)	1955	F	Severe	50	21–22	Normal	
23			F	Mild	25	21–22	Normal	
24	Soulier and associates (19)	1955	F	Severe	32	11	Abnormal (?)	
25	Stefanovic and associates (20)	1955	M	Severe	26	10	Abnormal	
26			M	Severe	27	13	Abnormal	
27	Chevallier and associates (21)	1955	M	Severe	45	12.5		
28			M	Severe	50	11		
29	Newcomb and associates (22)	1956	F	Moderate	17–50	15	Abnormal	
30	Telfer and associates (23)	1956	F	Moderate	28	11.2	Abnormal	Corrective
31	Jürgens (24)	1956	M	Severe	80	20	Normal	

* Work of present authors.
† Work of Ackroyd quoted and confirmed by Bergsagel and Hougie (41).

thrombin utilization in clotting blood was determined by the method described by Graham and associates (31). *Partial thromboplastin time* was determined by the method of Langdell, Wagner, and Brinkhous (33). In one experiment washed platelets were substituted for cephalin in this test. The platelets were prepared by differential centrifugation of citrated blood, suspension in a large volume of saline, recentrifugation, and resuspension in a volume of saline equal to one-fifth the original plasma volume. *Prothrombin concentration* was determined by the Iowa two-stage method as described by Wagner, Graham, Penick, and Brinkhous (34). Thrombin formation from partially purified human prothrombin was measured by the method of Biggs and Macfarlane (29). *Prothrombin time* was determined by the method of Quick (35). For some experiments this test was modified by increasing the total volume of reacting mixture to 0.4 ml. to allow the addition of test substances. In other experiments a sedimented platelet coagulant, Product II (43), or "Stypven" was substituted for brain extract. *Thromboplastin generation test* was performed by the technique of Biggs and Douglas (36). *pH of serum* in stability studies was adjusted to desired values with a glass electrode pH meter (Beckman) at 25° C by adding suitable quantities of 0.1 N HCl or 0.1 N NaOH. *Stuart factor concentration* of serum and eluates was determined by correction of the patient's plasma clotting defect in a modified prothrombin time test. To 0.1 ml. of patient's plasma was added 0.1 ml. of brain extract, 0.1 ml. of normal control serum variously diluted with patient's serum, and 0.1 ml. of 0.025 M $CaCl_2$. On these mixtures prothrombin times were determined in triplicate. From the average of the clotting times at each dilution a calibration curve was constructed by plotting prothrombin time against Stuart factor concentration, *i.e.*, the per cent normal control serum in the mixture. The concentration of Stuart factor in samples of normal serum or eluate exposed to varying pH's or temperatures was determined by interpolation from the calibration curve after substituting the test substance for control serum in the above system.

CASE REPORT

A white male farmer, R. S., now aged 36, has had frequent epistaxes and hematomata; hemarthroses have

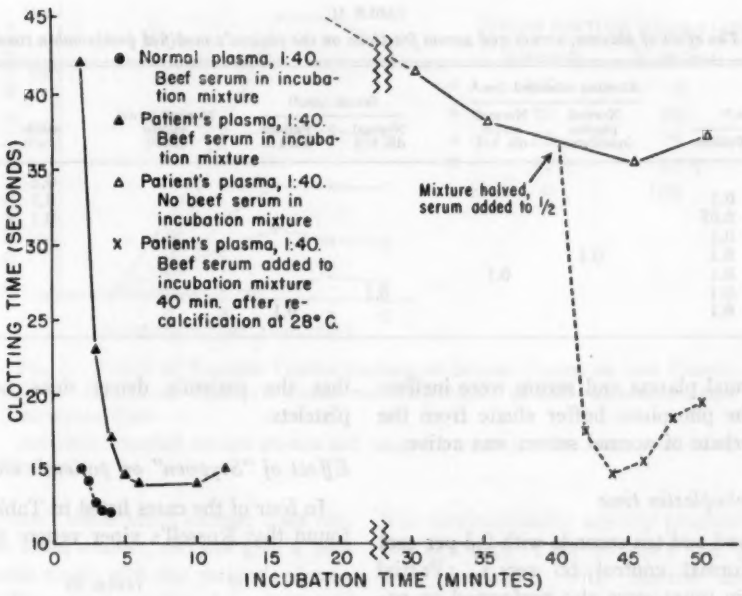

FIG. 1. THROMBIN EVOLUTION FROM PROTHROMBIN OF PATIENT'S PLASMA IN A TWO-STAGE PROTHROMBIN ASSAY WHEN SALINE WAS SUBSTITUTED FOR STORED BEEF SERUM IN THE INCUBATION MIXTURE CONTAINING LUNG THROMBOPLASTIN

All plasmas were diluted 1:40 before one part was added to three parts of the incubation mixture. At intervals 0.4 ml. of the mixture containing plasma and incubating at 28° C was added to 0.1 ml. of fibrinogen and the clotting times were recorded.

been infrequent and generally mild, but in December, 1955, there occurred a severe hemarthrosis of the right hip resulting in severe anemia. He received his first transfusion at this time, 1 unit of fresh blood, and apparently responded well. He now has five children with a sixth expected; the levels of the Stuart factor in the five tested varied between 21 per cent and 52 per cent of normal. Serum from the eldest son, aged 12, was used in an experiment described below. The boy's level of Stuart factor was 26 per cent of normal.

RESULTS

Study of the patient's blood, platelets, plasma and serum yielded the following results:

Whole blood clotting time

14 minutes (normal control 8 to 10 minutes).

Prothrombin concentration

Two hundred and twenty units, 86 per cent of control, when the two-stage incubation mixture contained stored beef serum. When saline was substituted for beef serum in the incubation mixture, only small amounts of thrombin were produced from the patient's prothrombin over a 40-minute period. The addition of beef serum after 40 minutes resulted in the production of almost as much thrombin as when beef serum had been present initially (Figure 1).

Prothrombin utilization

Forty-six per cent prothrombin remained 60 minutes after venipuncture, indicating significant impairment of prothrombin utilization. This confirms the earlier work of Lewis, Fresh, and Ferguson (9) who stressed that prothrombin utilization was abnormal in this patient.

Effect of normal plasma and serum and their fractions on patient's modified prothrombin time

It can be seen from Table II that the prolonged prothrombin time of the patient's plasma was corrected by normal plasma and serum. Alumina-

TABLE II

The effect of plasma, serum and serum fractions on the patient's modified prothrombin time

Plasma (*cm.*³)		Alumina adsorbed (*cm.*³)		Serum (*cm.*³)		Normal serum eluate (*cm.*³)	Saline (*cm.*³)	Modified prothrombin time (*sec.*)
Normal	Patient	Normal plasma (undiluted)	Normal serum dil. 1:5	Normal dil. 1:5	Patient dil. 1:5			
0.1							0.1	12.2
	0.1						0.1	85
0.05	0.05						0.1	12.8
	0.1					0.1		13.2
	0.1	0.1						59.0
	0.1		0.1					62.8
	0.1			0.1				12.6
	0.1				0.1			68

adsorbed normal plasma and serum were ineffective, while the phosphate buffer eluate from the alumina adsorbate of normal serum was active.

Partial thromboplastin time

Five hundred and ten seconds with 0.3 per cent cephalin (Normal control 66 secs.). Partial thromboplastin times were also performed on patient's and normal plasma using both patient's and normal washed platelets. The normal platelets clotted the patient's plasma in 137 seconds, the normal plasma in 75 seconds. Patient's platelets clotted the patient's plasma in 135 seconds and the normal plasma in 71 seconds. It would appear that Stuart factor, unlike Factor V (37), can be easily removed from normal platelet suspensions prepared from citrated blood. It also appears that the patient's defect does not lie in his platelets.

Effect of "Stypven" on patient's clotting defect

In four of the cases listed in Table I it has been found that Russell's viper venom gives a normal

TABLE III

Effect of "Stypven" on clotting defect of patient's plasma

Type of plasma		Brain extract (*cm.*³)	"Stypven" 10 mg./100 ml. (*cm.*³)	Saline (*cm.*³)	Modified prothrombin time (*sec.*)
Patient (*cm.*³)	Normal (*cm.*³)				
	0.1	0.1		0.1	12.2
0.1		0.1		0.1	85
	0.1		0.1	0.1	18.5
0.1			0.1	0.1	27
	0.1	0.1	0.1		7.5
0.1		0.1	0.1		22.5

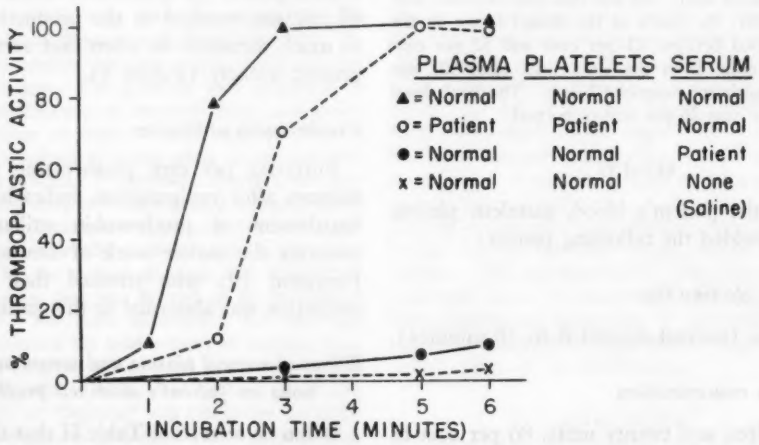

FIG. 2. THROMBOPLASTIN GENERATION TESTS PERFORMED ON MIXTURES OF VARIOUS REAGENTS PREPARED FROM PATIENT AND NORMAL CONTROL

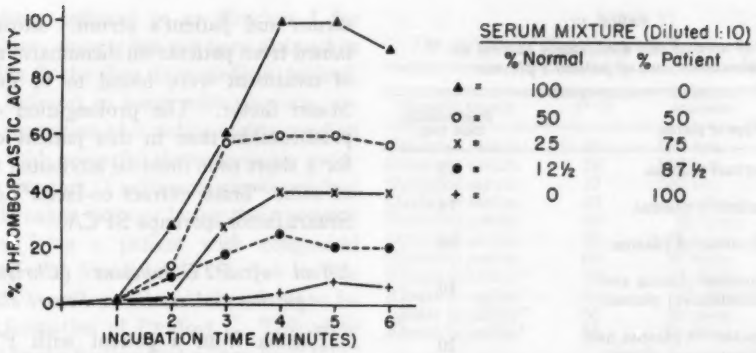

FIG. 3. EFFECT OF VARYING CONCENTRATIONS OF STUART FACTOR ON THE PRODUCTION OF COAGULANT ACTIVITY UNDER THE CONDITIONS OF THE THROMBOPLASTIN GENERATION TEST

$Al(OH)_3$ adsorbed normal plasma and washed normal platelets were used in all mixtures.

"prothrombin time" while brain extract does not. "Stypven," like brain extract, did not give a normal "prothrombin time" with our patient's plasma (Table III). This was true also of a mixture of "Stypven" and brain extract. "Stypven" plus cephalin, and "Stypven" plus platelets in both 1:10,000 and 1:20,000 concentration also failed to give a normal "prothrombin time" with the plasma of Crockett's (25) patient.

Thromboplastin generation test

The patient's alumina-treated plasma and his platelets were active in the thromboplastin generation test, but his serum was inactive (Figure 2). The thromboplastic activity produced when various mixtures of the patient's and normal serum were incubated with normal alumina-treated plasma, normal platelets and calcium is shown in Figure 3. It can be seen that the coagulant activity varied directly with the concentration of the Stuart factor. Prolonging the incubation period above 6 minutes did not result in a further increase in activity.

Effect of plasma and serum from dicoumarolized patients on Stuart clotting defect

Plasma from a patient obtained on the third day of dicoumarol therapy with a prolonged pro-

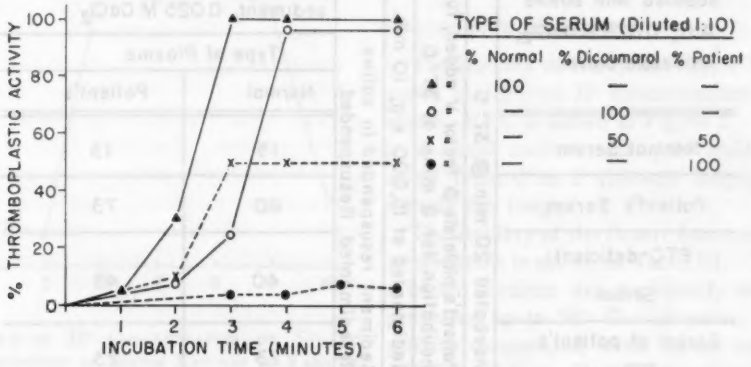

FIG. 4. EFFECT OF SERUM FROM A DICOUMAROLIZED PATIENT ON PATIENT'S SERUM IN THE THROMBOPLASTIN GENERATION TEST

$Al(OH)_3$ adsorbed normal plasma and washed normal platelets were used in all systems.

TABLE IV

Effect of normal and dicoumarol plasma on prothrombin time of patient's plasma

Type of plasma	Prothrombin time (*sec.*)
0.1 ml. normal plasma	13
0.1 ml. patient's plasma	73
0.1 ml. dicoumarol plasma	34
0.05 ml. normal plasma and 0.05 ml. dicoumarol plasma	19
0.05 ml. patient's plasma and 0.05 ml. dicoumarol plasma	20

thrombin time was found to correct the prothrombin time of R. S.'s plasma almost as well as normal plasma (Table IV). It should be emphasized that serum prepared at the same time from this dicoumarolized patient was normal in the thromboplastin generation test (Figure 4), and the two-stage prothrombin concentration of the plasma was 70 per cent of normal, despite a plasma prothrombin time of 34 secs. It can be seen that equal mixtures of serum from this dicoumarolized patient and Stuart's serum (Figure 4) gave the same 50 per cent thromboplastic effect as was shown in Figure 3 with equal mixtures of normal serum and patient's serum. Most samples obtained from patients on dicoumarol at a later stage of treatment were found to be deficient in the Stuart factor. The prolongation of the plasma prothrombin time in this patient on dicoumarol for a short time must be attributed to a deficiency of some "brain extract co-factor" other than the Stuart factor, perhaps SPCA.

Effect of PTC-deficient (Christmas disease) plasma and serum on Stuart clotting defect

Plasma from a patient with PTC deficiency (Christmas disease) corrected the prolonged prothrombin time of the patient's plasma to the same extent as normal plasma. Also a mixture of equal parts PTC deficient serum (1:10) with patient's serum (1:10) produced the same amount of thromboplastic activity in the thromboplastin generation test as normal serum at a 1:20 dilution.

Role of the Stuart factor in thromboplastin formation

Biggs, Douglas, and Macfarlane (40) have shown that a plasma fraction rich in AHF undergoes a preliminary reaction with serum and calcium ions to form an intermediate product. This

TABLE V

Effect of various types of serum on formation of sedimentable Product II *

Type of serum incubated with bovine AHF, 0.025 M $CaCl_2$, imidazole buffer	Incubated 20 min. @ 37° C	Patient's platelets & factor V added, further incubation for 5 mins. at 37° C	Sedimented at 15,000 x g, 10 min.	Sediment resuspended in saline. Resedimented. Resuspended.	C.T. (secs) of 0.1 ml platelet-poor plasma, 0.1 ml of once washed sediment, 0.025 M $CaCl_2$	
					Type of Plasma	
					Normal	Patient's
Normal Serum					15	13
Patient's Serum					60	73
PTC-deficient Serum					40	43
Serum of patient's son					23	23

* Mixtures consisted of 0.5 ml. bovine AHF (100 mg. per 100 ml.), 0.5 ml. imidazole buffer, 0.5 ml. of 0.025 M $CaCl_2$ and 0.5 ml. of each type of serum. Normal serum was diluted 1:20, the other sera 1:10. After preliminary incubation, 0.5 ml. of a suspension of patient's platelets and 0.2 ml. of Factor V were added to each mixture.

product has been referred to as Product I for convenience alone, since it has not been claimed or shown that this is the first intermediate formed. Product I is believed to react with platelets, resulting in the formation of a sedimentable product (Product II) with powerful thromboplastic properties. These Product II sediments can be washed at least twice in saline without losing their potency (41). Serum from a patient with congenital PTC deficiency was found to be inactive in this system, and PTC was considered, therefore, to be necessary for formation of Product I. This view was supported by the finding that a "purified" PTC preparation, prepared by the method of White, Aggeler, and Glendening (42) and containing less than 5 per cent Factor VII when assayed by the technique of Koller, Loeliger, and Duckert (3), could be substituted for normal serum.

An experiment was devised to determine the role of Stuart factor in the formation of Product II. A mixture consisting of AHF, imidazole buffer, and $CaCl_2$ was preincubated at 37° C separately with normal serum, the patient's serum, PTC deficient (Christmas disease) serum, or serum from the patient's son with only 26 per cent Stuart factor. "Product II" was prepared from each mixture as shown in detail in Table V, and the relative coagulant activities of the various washed and resuspended deposits were tested on platelet-poor plasma.

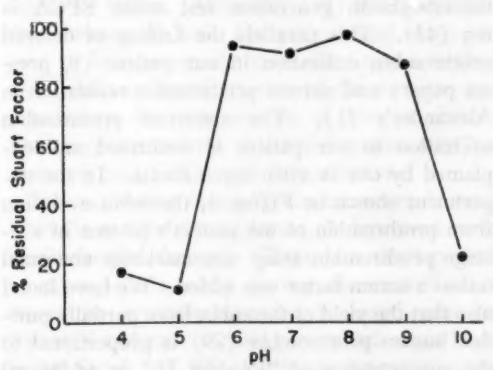

FIG. 5. EFFECT OF H^+ CONCENTRATION ON STUART FACTOR CONCENTRATION OF SERUM EXPOSED TO VARIOUS pH's 30 MIN. AT 37° C

The volumes of the control and various treated sera were adjusted to a common volume with 0.1 N NaCl after the pH's had been returned to 7.3.

TABLE VI

The stability of Stuart factor with time and temperature

Material treated	Temperature (° C)	Length of exposure	% residual Stuart factor
Oxalated serum	28	120 hrs.	78
Oxalated serum	28	168 hrs.	70
Oxalated serum	37	240 hrs.	25
Oxalated serum	45	6 mins.	99
Oxalated serum	56	2½ mins.	22
Oxalated serum	56	30 mins.	2
Oxalated serum	65	30 mins.	0
Eluate in saline*	50	30 mins.	100
Eluate in saline*	56	2½ mins.	82
Eluate in saline*	56	30 mins.	45
Eluate in saline*	65	30 mins.	29

* Eluate prepared by adsorbing oxalated serum with $BaSO_4$, 40 mg. per ml., eluting with 3.2 per cent sodium citrate, dialyzing against cold oxalated saline for 2 to 3 hours with three changes.

It can be seen that the Stuart factor, as well as PTC, was necessary for the formation of the active sediment. The sediment prepared from the serum of the patient's son showed that partial reduction in Stuart factor reduced somewhat the activity of Product II. The sedimentable thromboplastin prepared with normal serum, it should be noted, gave a normal clotting time with the patient's plasma despite the rinse in saline. Thus the sedimentable product had an effect not possessed by brain extract (Table II), lung thromboplastin (Figure 1) or washed normal platelets prepared from citrated plasma. It appears to be a fair assumption from this experiment that our patient's clotting defect lies primarily in the failure to form "blood thromboplastin" as in Christmas disease.

Properties of Stuart factor

The stability of the Stuart factor in serum exposed to different H^+ concentrations for 30 minutes at 37° C is shown in Figure 5. Stuart factor in a serum medium was quite stable in the pH range from 6 to 9 although largely inactivated outside this range.

The stability of the Stuart factor with time and temperature is shown in Table VI. The factor in a serum medium was moderately stable at temperatures up to 56° C. However, most of the activity disappeared when serum was heated at 56° C or higher. Heating the citrate eluate from barium sulphate adsorption of a sample of the same serum at 56° C caused much less loss of activity.

Effect of patient's plasma and serum on clotting defect of a case of congenital deficiency of SPCA

Lyophilized samples of the patient's plasma and serum were sent to Dr. Benjamin Alexander who found that the plasma of his patient (1) (congenital deficiency of SPCA by prior definition) and the plasma of our patient were mutually corrective in the prothrombin time test. Dr. Alexander has informed us that the thromboplastin generation test is normal in his patient (43). He has also found that both patients appear to lack "proconvertin" as measured by Owren's technique (6), and that a mixture of equal parts of the plasmas of the two patients appears to have 50 per cent "proconvertin" activity in the Owren assay. These data suggest strongly that the defects in the two patients should be considered distinct.

Effect of patient's plasma and serum on clotting defect of case of Crockett and associates

Crockett and associates (25) studied a patient (H. H.) with a congenital clotting defect. This patient had a prolonged prothrombin time which was corrected by normal serum. It can be seen (Table VII) that a normal prothrombin time was not obtained when equal parts of the plasma of the above patient and the plasma of R. S. were mixed, although mixture with normal plasma was successful with both patients. It was shown also that the two sera were not mutually corrective in the thromboplastin generation test, an equal mixture of each being inactive. The defects in the two cases, therefore, can be considered the same.

TABLE VII

The effect on patient's plasma, of normal plasma and the plasma of a previously described patient with a clotting defect

Plasma			Prothrombin time (*sec.*)
R. S.	H. H.	Normal	
		0.1	12.2
	0.1		38
0.1			60
0.05	0.05		44
	0.05	0.05	13.4
0.05		0.05	13

DISCUSSION

The direct mixing of the plasmas of our patient and Alexander's with mutual correction of the prolonged prothrombin times, plus the completely different action of the two in the thromboplastin generation test indicate that the two defects are not identical. Thus, a patient diagnosed as having SPCA deficiency and one diagnosed as having proconvertin deficiency have different defects. This throws open the whole question of the action of these factors and requires that the literature be re-evaluated in the light of this finding. The puzzling inconsistencies in Table I with regard to thromboplastin generation and "Stypven" action are probably explained by heterogeneity of the cases.

It would be interesting to know the effect of "Stypven" on the plasma of Alexander's patient. The fact that "Stypven" failed to correct the defect of both our patient and Crockett's, yet corrected the defects of Hicks' (16) and Jenkins' (38) patients with Factor VII deficiency, Hjort, Rapaport, and Owren's with hypoproconvertinemia (39) and Telfer, Denson, and Wright's with "Prower factor" deficiency (23) suggests that "Stypven" might prove useful in categorizing bleeders with a prolonged prothrombin time due to absence of one of the "stable" factors.

It is of great interest that the Stuart factor is required for "thromboplastin" formation in the thromboplastin generation test while SPCA is not (43). This parallels the finding of delayed prothrombin utilization in our patient (9, present paper) and normal prothrombin utilization in Alexander's (1). The abnormal prothrombin utilization in our patient is confirmed and explained by our *in vitro* experiments. In the experiment shown in Figure 1, thrombin evolution from prothrombin of the patient's plasma in a 2-stage prothrombin assay was markedly abnormal unless a serum factor was added. We have found also that the yield of thrombin from partially purified human prothrombin (29) is proportional to the concentration of "Product II," in additional experiments not included in the present communication. Thus there appears to be a direct relationship between Stuart factor concentration and activity of Product II on the one hand, and concentration of Product II and the yield of

thrombin from prothrombin on the other. The prothrombin utilization defect in our patient appears secondary to defective formation of blood thromboplastic activity.

The experiments with the washed sediments (Table V) show clearly that the Stuart factor is as necessary as AHF and PTC for the formation of the early intermediate, Product I, which appears to unite in some manner with platelets to form a sedimentable thromboplastin, Product II. The sedimentable Product II, after a wash in saline, gives the same normal "prothrombin time" with the patient's plasma as with normal plasma, suggesting from another direction that the patient's defect in conversion of prothrombin to thrombin is the reflection of a defect in the formation of a "complete" blood thromboplastin. The facts that washed normal platelets alone do not correct the patient's defect while the washed Product II prepared with normal serum does correct it, seem to imply that the Stuart factor in the Product II sediments is more closely bound to platelets than mere occluded plasma.

Retrospectively, it would appear that the chief reason for the assumed identity of Factor VII, SPCA, and proconvertin has been the wide use of assays of the Koller (3) and Owren (4) types. The substrate for both methods consists of plasma filtered through asbestos. This substrate is known to contain most of its original prothrombin but has been thought to be deficient in only a single accessory factor. Alexander (43) has found that the plasmas of both his patient and ours appear to lack proconvertin by the Owren method (4), while an equal mixture of the two has approximately half the activity of normal plasma. This suggests that the asbestos filtration step has removed both SPCA and Stuart factor.

It is interesting that a "brain extract co-factor," which is not the Stuart factor, is depressed early in dicoumarol therapy. There is a short period, in other words, early during dicoumarolization when the prothrombin time is lengthened yet Factor V and prothrombin concentration are high, Stuart factor and PTC levels are not significantly reduced, and the thromboplastin generation test is normal. Later during therapy the Stuart factor becomes reduced along with PTC (44–46) and prothrombin (47).

The dicoumarol experiments raise the question whether the Stuart factor is identical with the postulated new factor, Factor X (48) since the Factor X effect was first noted in dicoumarol plasma. Factor X appears to be less stable than the Stuart factor, since Factor X is said to disappear in a few hours at room temperature (48), and the Stuart factor is stable much longer under similar conditions. Moreover, Factor X in concentrations varying between 1 per cent and 100 per cent is thought to affect the velocity of blood thromboplastin formulation but not the final yield (48), although in concentrations lower than 1 per cent, thromboplastin generation may be almost impossible (48). Our experiments show that the amount of coagulant activity produced in the thromboplastin generation test is related directly to Stuart factor concentration. It would appear that the Stuart factor is not the same as Factor X.

The pH and storage stabilities of Stuart factor are pronounced for a clotting factor and almost identical with those shown for SPCA by de Vries, Alexander and Goldstein (2). Since it has been demonstrated that Alexander's patient and ours have different defects, this poses a serious problem in interpretation. There are at least two possible explanations for the similarity of the properties of SPCA and Stuart factor. Either the two factors have very similar physical and chemical though different physiological properties, or de Vries and associates (2) were measuring Stuart factor in their SPCA assay rather than the factor which their patient (later described) was found to lack. It would be very interesting to compare the results obtained if their experiments were repeated, using the genetically deficient SPCA plasma as the test substrate alongside the original assay.

Heretofore, it has been agreed that the factors which are clearly essential for a normal thromboplastin generation test (AHF and PTC) have no effect on prothrombin time. Also, the "stable" factor essential for a normal prothrombin time (SPCA, Factor VII) has been recognized as having an equivocal relation to thromboplastin generation. The Stuart factor appears to be necessary for both a normal prothrombin time and for a normal thromboplastin generation test. This is disturbing because the characteristics of Stuart factor cut across our usual thought categories. It

raises the question whether the Stuart defect is not, in reality, a double deficiency. This question cannot be answered categoricaliy at present, because of the omnipresent possibility of undescribed factors. However, it seems unlikely for two reasons. It has been shown previously that Stuart's plasma (then thought to be SPCA-deficient) corrects the prolonged partial thromboplastin time of plasmas from classic hemophilia, PTC-deficiency, Ac-globulin deficiency, and PTA deficiency (49). Alexander has shown that it also corrects SPCA deficient plasma (43), and it does not have the characteristics of Hageman factor (50) or Factor X (48). The hypothesis of double deficiency would imply under these circumstances that the plasma is deficient in *two new* factors. The principle of economy of hypotheses suggests that it might be wise to invoke only one new factor at this time. Secondly, if the conventional genetic assumption that each of these deficiencies results from a mutant gene at a specific and unique locus is made, the probability of a double deficiency can be shown to be exceedingly small.

It is not possible at present to decide with certainty which of the reported cases of SPCA, Factor VII and proconvertin deficiency probably match Alexander's patient and which ours. Retesting all of the patients with both the thromboplastin generation test and "Stypven" would be helpful. However, mutual exchange and cross-matching in several clotting systems appears to be the ultimate test. Of the patients in the literature (other than Crockett's), our patient's defect more nearly resembles that of de Vries' patients (15), Stefanovic's (20), Newcomb's (22) and Telfer's (23), the ones having abnormal thromboplastin generation tests. However, and this may be crucial, the Prower defect of Telfer's patient is corrected by "Stypven" in marked contrast to both our patient and Crockett's. This may well mean that the Stuart and Prower defects are different. The absence of tests with "Stypven" does not allow one to speculate further about the others. It is possible also that Quick, Pisciotta, and Hussey (13) have cases of both Stuart factor and SPCA deficiency amongst their patients with prolonged prothrombin times but showing mutual correction. At the moment, this possibility is obscured by the lack of two-stage prothrombin data as well as thromboplastin generation and "Stypven" tests. We would like to suggest, and are prepared to cooperate ourselves, that the workers who have reported cases of "stable factor" deficiency exchange lyophilized samples of plasma and serum in an attempt to categorize these patients exactly.

CONCLUSIONS

1. A patient was re-studied who had been diagnosed previously as hypoproconvertinemia. He had an abnormal thromboplastin generation test, and his defect was not corrected by "Stypven."

2. The deficient factor was shown not to be SPCA by cross-matching and is being called the *Stuart factor* after the patient's surname.

3. Stuart factor has been found to be essential for the formation of "blood thromboplastin."

4. Stuart factor has unusual actions, being necessary early in "blood thromboplastin" formation and required for optimal activity of brain, lung, and platelet thromboplastins, cephalin and "Stypven."

5. The concentration of Stuart factor has been found to be high early in dicoumarol therapy, despite a prolonged prothrombin time, but to be diminished later.

6. Stuart factor is relatively heat and pH stable.

7. Stuart factor can be separated from platelets by a single saline wash, but is not removed from the sedimentable coagulant, "Product II," by a similar procedure.

8. Assay procedures for "proconvertin" and "Factor VII" using asbestos-adsorbed plasma as substrate are probably sensitive to changes in the levels of both SPCA and Stuart factor.

9. The hemorrhagic state(s) previously classified as congenital "hypoproconvertinemia," or "SPCA deficiency" or "Factor VII deficiency" are probably not identical diseases. There are at least two separable conditions included in this group.

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STUART CLOTTING DEFECT. II. GENETIC ASPECTS OF A 'NEW' HEMORRHAGIC STATE

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The literature is ambiguous regarding patients deficient in the clotting factor variously known as "SPCA," "proconvertin," and "Factor VII." Sera from some patients are normal in the thromboplastin generation test; those from others are abnormal. Viper venom corrects the clotting defect in some cases, but fails to do so in others. Despite these contradictions, it has been generally assumed that the terms are synonyms for a single clotting factor with unique characteristics.

In our preceding communication on the patient R. S. (1), previously reported by others as hypoproconvertinemia (2), we pointed out that the assumption of identity must be incorrect, since the plasmas of our patient and the SPCA deficient patient of Alexander, Goldstein, Landwehr, and Cook (3) were mutually corrective, while our patient's plasma failed to correct that of the patient of Crockett, Shotton, Craddock, and Leavell (4). This finding implied the existence of at least two $BaSO_4$ adsorbable clotting factors whose lack prolongs the prothrombin time. We showed that, in contrast to certain other cases in the literature (5–8), the defect in our patient's plasma could not be corrected by Russell viper venom, and that his serum was inactive in the thromboplastin generation test. In this last respect our patient's defect resembled that of some of the previously reported cases (8–11) but differed from certain others (5, 6, 12–14).

The factor deficient in our patient is being referred to as the Stuart factor after the patient's surname. We wish to emphasize by this nomenclature that only by cross-matching his plasma with that from other similar patients can an identity be definitely established. The properties of Stuart factor and its role in blood coagulation were the subject of our earlier communication (1). In this paper the studies on the relatives of our patient will be reported. Our genetic studies indicate that the Stuart defect is inherited as a highly penetrant, incompletely recessive, autosomal characteristic.

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MATERIALS AND METHODS

Blood for study was obtained from certain members of the family on three separate occasions. On the first field trip, blood was obtained from the proband, his wife, and two of his sons. On the second field trip, blood was obtained during the course of a single day from all the persons shown in Table I. On the third trip, blood was obtained from the proband, his wife, and his daughter. It was established on the first trip that the plasma and serum of the proband's wife did not differ significantly in concentration of Stuart factor from several, presumably normal, laboratory workers. Henceforth, the wife was used as the control subject, in the belief that a field control was required to cover the manipulations of venipuncture, transportation and storage. On all occasions, blood was obtained by the two syringe, silicone technique and was centrifuged in a portable Servall centrifuge in siliconed centrifuge tubes.

Plasma was pipetted immediately from the centrifuge tubes into storage tubes; prothrombin time was determined at once on a sample, and the remainder was quickly frozen and stored at — 70° C. The following day, prothrombin times and Stuart factor assays were performed on all samples of plasma in our own laboratory.

Serum was obtained from the members of the family at the same venipuncture as plasma. Whole blood was placed in clean, non-siliconed centrifuge tubes. Tubes were stoppered and the blood allowed to clot, precautions being taken to prevent hemolysis. The clotted blood remained at automobile and room temperature for 18 to 21 hours before centrifugation. The serum was expressed by centrifugation in our own laboratory the next day and tested for residual prothrombin and thrombin. When a serum sample contained detectable prothrombin or thrombin it was placed in a 28° C waterbath until the clotting time with thromboplastin plus fibrinogen was greater than 300 seconds, and it did not clot fibrinogen in 10 minutes. This additional incubation was required in only a few cases, notably the proband's. All samples of serum were frozen and stored at — 20° C, including the control serum from the proband's wife. All thromboplastin generation tests were performed on the second and third day after obtaining the serum. The same $Al(OH)_3$-adsorbed normal plasma and 0.03 per cent

TABLE I

The plasma and serum Stuart factor levels of fourteen members of the Stuart kindred

Number	Relation to proband	Per cent of Stuart factor			Probable genotype
		Prothrombin time method	Thpln generation method	Average	
IV-28	Proband	0*	0*	0	Homozygous abnormal
III-11	Mother	47	40	44	Heterozygous
III-23	Paternal uncle	44	45	44	Heterozygous
III-25	Paternal aunt	52	37	44	Heterozygous
V-37	Son	32	20	26	Heterozygous
V-38	Son	32	22	27	Heterozygous
V-39	Son	11	31	21	Heterozygous
V-40	Son	32	26	29	Heterozygous
V-41	Daughter	44	60	52	Heterozygous
III-3	Maternal aunt	100	100	100	Homozygous normal
III-5	Maternal uncle	93	80	86	Homozygous normal
III-21	Paternal uncle	100	100	100	Homozygous normal
IV-7	Aunt by marriage	100	100	100	Homozygous normal
IV-26	Wife	100	100	100	Homozygous normal

* One to three per cent according to Lewis, Fresh, and Ferguson (2).

cephalin suspension (in lieu of platelets) were used in all generation tests.

Stuart factor assays

Plasma method. The assay procedure was based on a comparison of the correction by control and test plasma of the proband's prolonged prothrombin time. The plasma of the proband (prothrombin time 60 to 85 seconds) was the substrate for the test. Normal plasma (prothrombin time 12 to 14 seconds) was diluted with the patient's plasma 1:2 through 1:16. Prothrombin time (15) was determined on the various mixtures in triplicate and the values at each dilution were averaged. A calibration curve was constructed by plotting prothrombin time against concentration of the Stuart factor, *i.e.*, the percentage of normal plasma in each mixture, the 1:2 dilution representing 100 per cent.

An unknown plasma was assayed after being diluted 1:2, 1:4, and 1:8 with patient's plasma. Prothrombin times were performed on each dilution in triplicate and averaged. Per cent Stuart factor was interpolated from the calibration curve for each dilution of the unknown plasma. The percentage value from the 1:4 dilution was doubled, the value from the 1:8 dilution quadrupled, and both were averaged with the value from the 1:2 dilution. The final average value for each plasma is shown in Table I.

The arbitrary 100 per cent value in this test is actually equivalent to 50 per cent Stuart factor. An unknown plasma with 50 per cent Stuart factor is, therefore, one which diluted 1:2 with Stuart's plasma, has a clotting time equal to that of the normal control diluted 1:4. (A typical dilution curve on normal plasma, November 27, 1955, was as follows: 1:2(100%)–14.4 secs., 1:4 (50%)–16.5 secs., 1:8(25%)–19.6 secs., 1:16(12.5%)–24 secs.)

Serum method. The Stuart factor content of an unknown serum was determined in the thromboplastin generation test (16) after it had been established that the proband's serum was only about as effective as saline in this test (1). Normal serum was diluted in a serial two-fold fashion 1:2 through 1:8 with proband's serum prior to diluting 1:10 with normal saline for use as the serum source in the generation test. The calibration curve was constructed by plotting the minimum clotting time at each dilution (at whatever incubation time) against Stuart factor concentration, *i.e.*, the per cent normal serum in the mixture.

Unknown sera were assayed by diluting 1:2, 1:4, and 1:8 with patient's serum then 1:10 with saline and determining the minimum clotting time in the thromboplastin generation test (at whatever incubation time). Per cent Stuart factor in each dilution of each unknown was determined by interpolation from the calibration curve. The percentage Stuart factor obtained at the 1:4 dilution was multiplied by 2 and that at 1:8 by 4. All three percentage values were averaged and are the averages shown in Table I.

In this assay the 1:2 dilution of normal serum with patient's serum was arbitrarily designated 100 per cent on the calibration curve. A value of 50 per cent or less for an unknown serum implies that it had the same or less activity than normal plasma carried one dilution step further. (A typical dilution curve of control serum with patient's serum on November 29, 1955, showed the following minimum clotting times: 1:2(100%)–14.5 secs., 1:4 (50%)–17 secs., 1:8(25%)–22 secs.)

PEDIGREE

The proband belongs to a large kindred living in the Blue Ridge mountains of the northwestern corner of North Carolina and nearby southwestern Virginia. Information was obtained on 164 members of the family; approximately 100 still reside

within a 30-mile radius of West Jefferson, North Carolina.

The pedigree is shown in Figure 1. The proband (IV-28), a moderately severe bleeder and symptomatic since birth, had not required transfusions until December, 1955, at age 36. His relatives were quite dogmatic that only he should be considered a bleeder. Closer questioning of his wife, however, disclosed that one of his sons (V-39) seemed to have unusually frequent nosebleeds, and that the buttocks of his daughter (V-41) often showed persistent bruises after disciplinary spanking. No history of even a mild bleeding tendency could be elicited from other members of the kindred.

The proband works as a tenant farmer, farm laborer, and lay preacher in the periods between hemorrhagic crises, never dangerous until the latest one. Exacerbations have been of such frequency and irregularity that he has been unable to retain regular employment and has found it difficult to support his family. His chief symptoms have been excessive bleeding from small cuts, occasional hemarthoses and persistent anemia.

It can be seen in Figure 1 that our proband was born of a consanguineous union. His mother (III-11) and father (III-12) were related to each other as aunt and nephew, a not unknown mating type 40 years ago in this isolated mountain area. It should be noted also that there is another but less close consanguineous mating in the pedigree (III-5 to IV-7), in which the abnormal genes were probably absent as judged by assay procedures and 13 apparently normal children.

We assayed for the Stuart factor both plasma and serum from the 14 persons needed to establish a genetic hypothesis. Those available included the proband's mother, children and wife, two of the mother's siblings, and three siblings of his deceased father. The results are shown in Table I. It can be seen that the levels of Stuart factor found with the two procedures agree fairly well. If the values from the two procedures are averaged they fall clearly into three distinct groups. There is an intermediate class between the very low level of the proband (1 to 3 per cent according to Lewis and associates [2]) and the normal persons, such as his wife. The mean value for Stuart factor in this intermediate group is 36 per cent with a standard deviation of 9.6 per cent. It is logical, therefore, to hypothesize that the proband is homozygous for an abnormal autosomal gene, that the intermediate class is heterozygous, and that the persons with normal levels are homozygous normal.

This hypothesis can be tested for internal consistency by analyzing the distribution of the heterozygous and normal individuals.

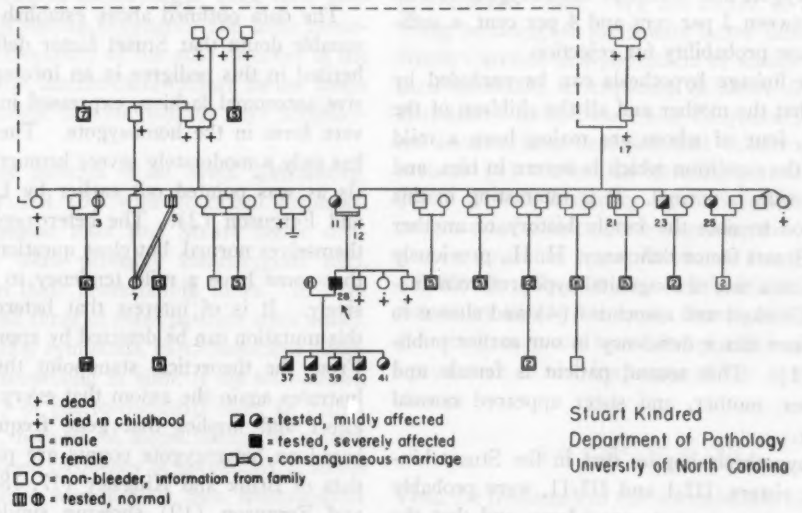

FIG. 1. THE STUART KINDRED

1. The mother of the proband, all his children and two siblings of his (deceased) father are heterozygous as classified by our tests.
2. Both father and mother of the proband have normal siblings.
3. The proband's wife is normal, and there are no homozygous children.
4. The heterozygous siblings of the proband's father, who did not mate consanguineously, do not have clinically affected children.

All these facts are consistent with the hypothesis that the proband is homozygous for an incompletely recessive autosomal gene (*st/st*), his mother, two of his father's siblings and all his children are heterozygous (*St/st*), and his maternal aunt, maternal uncle, paternal uncle, aunt by marriage and wife are homozygous normal (*St/St*).

Another hypothesis which might fit these data is that an autosomal gene for the Stuart defect is completely expressed in some individuals in the heterozygous condition (the proband) but very slightly expressed in others (his mother and children). This can be rejected by observing that all five of the proband's children would be required to have received the abnormal gene from their father. If his wife, genetically unrelated and normal by test, were in fact normal (a reasonable assumption), the probability that every child of a heterozygote also would be heterozygous is 1 in 32 or between 3 per cent and 4 per cent, a sufficiently low probability for rejection.

A sex linkage hypothesis can be excluded by noting that the mother and all the children of the proband, four of whom are males, have a mild form of the condition which is severe in him, and that his wife is normal. It is interesting in this connection to note the family history of another case of Stuart factor deficiency, H. H., previously reported as a case of congenital hypoprothrombinemia by Crockett and associates (4) and shown to have Stuart factor deficiency in our earlier publication (1). This second patient is female and her father, mother, and sister appeared normal clinically.

Our hypothesis implies that in the Stuart kindred the sisters, III-1 and III-11, were probably heterozygous at an autosomal locus and that the man, now deceased, who was III-1's son and III-11's husband was also heterozygous. The consanguineous mating of III-11 and III-12 produced at least one homozygote, the proband (IV-28). No conclusions can be drawn about the proband's siblings since two died in infancy and the third died of a cerebral vascular accident which might have resulted either from hemorrhage or thrombosis.

Under our hypothesis, one-half of the members of generation III and all of the proband's children should be heterozygous. Reference to Figure 1 will show that this prediction is borne out precisely. Six members of the third generation and all the children were tested. Three of the six adults were clearly heterozygous as were all the children. These facts mean that, assuming our hypothesis to be correct, the abnormal gene is not only incompletely recessive but is also highly penetrant.

Finally, it should be noted that the persons with intermediate levels of the Stuart factor and scored as heterozygous, had 1½ to 3-second prolongations in the classical prothrombin time test and minimum clotting times in the routine thromboplastin generation test (serum phase) 2 to 3 seconds longer than control sera.

DISCUSSION

The data outlined above establish beyond reasonable doubt that Stuart factor deficiency is inherited in this pedigree in an incompletely recessive autosomal fashion, expressed in its most severe form in the homozygote. The homozygote has only a moderately severe hemorrhagic diathesis as was pointed out earlier by Lewis, Fresh, and Ferguson (2). The heterozygotes consider themselves normal, but close questioning discloses that some have a mild tendency to bleed excessively. It is of interest that heterozygosity for this mutation can be detected by appropriate tests. From the theoretical standpoint this finding illustrates again the axiom that every gene has an effect and implies that gene frequency studies based on heterozygote counts are possible. The data of Brink and Kingsley (17, 18) and Lewis and Ferguson (19) showing similar effects in

persons heterozygous for Factor V deficiency have, perhaps, similar implications.

From the practical standpoint, our data (and the data on Factor V) imply, as Quick has frequently reiterated (see Ref. 20), that a 1½ to 3-second prolongation of the prothrombin time should not be shrugged off automatically as a "vagary of the one-stage" provided that it occurs consistently and that the control is reproducible from day-to-day; we would add that a similar occurrence with the thromboplastin generation test may have a similar significance. The observer may be dealing in either instance with an individual heterozygous for an abnormal gene affecting one or another of the prothrombin accessory factors. The obvious conclusion is that all such plasmas should be assayed specifically for the possible deficiencies.

The fact that the heterozygotes in the Stuart kindred did not consider themselves symptomatic does not imply that this is always true. We have recently studied another kindred reputed to be "female bleeders." We discovered that roughly half the females examined had 1 to 3-second prolongations of plasma prothrombin time and 40 to 60 per cent Stuart factor levels as measured by the assays used in the present communication. These women were known to be bad operative risks by their physicians and to be persistently anemic, presumably because of menorrhagia, since their anemia improved during pregnancy and after menopause. The frequency of the mild disorder in the family and the intermediate Stuart factor levels led us to conclude that the patients were heterozygous.

If the three patients of de Vries, Kettenborg, and van der Pol (9) with abnormal thromboplastin generation tests had Stuart factor deficiency, they were probably heterozygous also, judging from their prothrombin times. It is of interest that all three of these patients were males. Their presenting symptoms, gastrointestinal bleeding, are not surprising in view of the well-known excess (4:1) of gastro-duodenal ulcer in males. Nevertheless, because of menstruation in women and the increased frequency of operations, it seems likely that more women than men heterozygous for the autosomally transmitted clotting dyscrasias will be referred to the laboratory as doubtful bleeders.

One member of the Stuart kindred, III-5, created a classification problem. However, he was scored as normal since his Stuart factor level (86 per cent) was more than 3 standard deviations greater than the mean of the indubitable heterozygotes. This conclusion could have been tested further by examining his 13 children, as half of them would be expected to be heterozygous under our hypothesis if the father were in fact heterozygous. These studies were not carried out, primarily because whether or not he is heterozygous does not affect the main hypothesis. Also since he lives 200 miles from our laboratory in a remote mountain area, testing all his children in the field constitutes a separate enterprise. However, study of his descendants is intended eventually, since it is of more than passing interest to discover whether heterozygosity for the Stuart defect can be present in a person with a normal level of Stuart factor. We have also not yet attempted to study the turnover rate of the Stuart factor in the proband, or the steady-state relationships in the heterozygotes. It is anticipated that these studies will be performed in the future.

A rough calculation of the frequency of the abnormal Stuart gene will be made, because it emphasizes our point about consistently prolonged prothrombin times. We are aware of only one living homozygote for Stuart factor deficiency in North Carolina in a population of somewhat more than 4,000,000, a frequency of about .00000025. However, Lewis, Fresh, and Ferguson (2) studied another, now deceased, and Crockett and associates (4) studied an identical patient in Virginia. If this frequency figure is quadrupled to compensate for incomplete ascertainment, it should give a *probable upper limit* of the frequency (.000001). Then if the Hardy-Weinberg conditions are assumed to apply, the upper limit of the gene frequency can be estimated efficiently as the square root of the frequency of the homozygous class or

$$q = \sqrt{.000001} = .001$$

Then the frequency of the normal allele can be obtained as $p = (1-q)$ or $(1-.001) = .999$ and the

frequency of heterozygotes as

$$2pq = 2(.999)(.001) = .0019$$

or about 2/1,000. This surprisingly high heterozygote frequency implies that if one surveyed a random population of North Carolinians by the prothrombin time test he might encounter as many as two persons in each thousand with prothrombin time consistently prolonged 1½ to 3 seconds where the prolongation resulted from heterozygosity for Stuart factor deficiency.

Finally, the demonstration that the abnormal autosomal gene for Stuart factor deficiency is incompletely recessive implies that the normal allele is incompletely dominant. This means biochemically either that a single normal allele at the Stuart locus does not furnish enough enzymatic activity or substrate at some point in the synthetic metabolic pathway to maintain the level of the factor at that of "wild type," or that the mutant gene inhibits some step (21). The normal allele at this locus is therefore, in its overall effect, like that for Factor V in some pedigrees (18, 19), some pedigrees of Christmas disease (22, 23), and some pedigrees of mild hemophilia (24), and unlike the allele for classic hemophilia (22, 23, 25).

SUMMARY

1. Stuart factor deficiency, recently segregated from the heterogeneous group of hemorrhagic states known variously as SPCA, proconvertin or Factor VII deficiency, has been studied in a large North Carolina kindred and shown to be inherited as a highly penetrant but incompletely recessive autosomal characteristic.

2. The heterozygotes have been found to be only mildly affected or normal clinically, but to have 1½ to 3-second prolongations of the prothrombin time and thromboplastin generation tests. These effects are, presumably, due to the reduction in the level of this factor to a mean of 36 per cent in the range 20 to 52 per cent.

3. It is pointed out that carrier detection may be possible in many instances with fairly simple tests.

4. It is emphasized that heterozygotes for the various hemorrhagic states, especially females, may be encountered as patients with abnormal operative bleeding, persistent gastro-intestinal bleeding or menorrhagia with persistent anemia. Such symptoms, in the absence of clean-cut laboratory evidence, however, should not be assumed to represent heterozygosity.

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AGE DIFFERENCES IN THE INTRAVENOUS GLUCOSE TOLERANCE TESTS AND THE RESPONSE TO INSULIN

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In addition to providing information with regard to the status of carbohydrate metabolism, the time course of the disappearance of injected glucose from the blood stream offers information concerning the overall effectiveness of a variety of physiological mechanisms involved in maintaining homeostasis. In a number of investigations, reduction in glucose tolerance, *i.e.*, a slower rate of return to fasting levels of blood sugar following the oral (1–17) or intravenous (18–20) administration of glucose, has been reported in older people. The diminished glucose tolerance in the older individual might be due to: (a) inadequate release of insulin from the pancreas, or greater inactivation of endogenously released insulin, (b) the loss of functioning protoplasm with increasing age so that less metabolizing tissue is removing glucose from the blood, (c) a diminution in the effectiveness of the metabolic processes involved in the removal of sugar from the blood stream, (d) alterations in the rate of release of glucose from the liver, or (e) a reduction in the volume in which the glucose is originally distributed in the aged. By comparing the glucose and glucose-insulin tolerance tests in the same individual, an estimate of the effect of insulin may be obtained (21–27). In the experiments to be reported, standard amounts of insulin were administered along with glucose to both old and young subjects with the aim of investigating age differences in the response to the insulin.

EXPERIMENTAL METHODS

Subject selection. Thirty-five male subjects, age 23 to 86 years, were selected on the basis of a detailed history, physical examination and a series of laboratory tests. The presence of any of the following served to exclude a subject from the study: (a) history or known evidence of diabetes or glycosuria, (b) severe alcoholism, hepatomegaly, cirrhosis or other liver disease, (c) cardiac decompensation or edema, (d) infections, temperature elevation or acute or chronic trauma (including surgical) within one week of test, or (e) the taking of steroid drugs (other medication such as aspirin was omitted for 12 hours preceding the tests). All were ambulatory in-patients on a routine full hospital diet for at least one week. Fasting blood sugars were within normal limits (27, 28) as shown in Table I.

Experimental procedure. The intravenous glucose tolerance test (GTT) and the glucose-insulin tolerance test (GITT) were performed in each subject under basal conditions and separated by an interval of not less than one week. In 8 of the subjects, each of the tests (GTT and GITT) was carried out twice in order to evaluate reliability.

Twenty minutes before either test was begun, a modified Lindeman needle was placed in an antecubital vein and left in place for the duration of the test. The needle was kept patent by heparinization of the stylus and was used subsequently only for withdrawing blood specimens without a tourniquet (29). A vein in the opposite arm was used for the injection of 50 ml. of 50 per cent glucose in water over a period of two minutes. For the GITT, 5 units of hyperglycemic factor-free insulin[3] (Lilly), per square meter of body surface area, were rapidly injected, followed immediately by the standard amount of glucose. The fasting blood specimen was obtained through the Lindeman needle a few minutes before zero time, which was recorded as the beginning of the injection of glucose.

Blood samples were collected at 5-minute intervals for the first hour and at 20-minute intervals during the second hour, and were placed immediately in tubes containing a dried heparin and sodium fluoride mixture. All analyses were completed the day of the test, using the Nelson-Somogyi method (30). Determinations were in duplicate and were read on a Model DU Beckman Spectrophotometer.

Data analysis. For each tolerance test, the observations obtained between 10 and 60 minutes of the experiment were fitted to the equation $\log_e y = \log_e A - kt$ ($y = Ae^{-kt}$) where y is the blood glucose concentration in

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[3] We are indebted to Dr. W. R. Kirtley of Eli Lilly and Company for supplying us with HGF free Z.I.C. 40 units per ml.

TABLE I

Subject characteristics

Variable		Age group			
		Young	Middle	Old	Total
N		12	11	12	35
Age (*yrs.*)	Mean	31.3	49.1	78.7	53
	Range	23	42	65	23
		37	58	87	87
Height (*cm.*)	Mean	175.3	174.4	162.6	170.8
	σ_{Mean}	2.0	2.7	2.3	2.2
	Range	165.7	165.1	143.5	143.5
		185.4	186.7	171.4	186.7
Weight (*Kg.*)	Mean	71.0	64.6	66.8	67.5
	σ_{Mean}	2.4	3.5	3.9	3.2
	Range	57.3	52.0	45.1	45.1
		85.0	92.7	88.2	92.7
Surface area ($M.^2$)	Mean	1.83	1.77	1.70	1.77
	σ_{Mean}	.033	.047	.060	.040
	Range	.67	1.58	1.39	1.39
		2.00	2.08	2.00	2.08
Fasting blood glucose*	Mean	68.0	78.5	85.0	77.1
	σ_{Mean}	1.9	2.0	2.3	2.1
	Range	60	64	70	60
		77	90	97	97
Glucose administered (*Gm./Kg. body wt.*)	Mean	.356	.397	.389	.381
	σ_{Mean}	.012	.018	.024	.020
	Range	.294	.270	.283	.270
		.436	.454	.554	.554

* Mean of control observations on glucose and glucose-insulin tests.

milligrams per 100 ml. and t is time in minutes, following the injection of the glucose load. The method of least squares (31) was utilized for the computation of A and k for each experiment. The value of k was taken as the index of tolerance for this study. The difference between the k for the glucose (k_G) and the glucose-insulin test (k_{GI}) is called Δ k and served as the index of the response to insulin in each subject. Visual fits to plots of log of the glucose level against time were also made and compared with the least squares fitting.

Age changes in the data were evaluated by determining the regression of the derived measures on age and also by comparing mean values for three groups: young (12 subjects, age 20 to 39 years), middle (11 subjects, age 40 to 59 years), and old (12 subjects, age 60 to 90 years).

RESULTS

Characterization of the subjects

No significant ($P = > 0.10$) differences were found among the three age groups with respect to body weight, surface area or dose of glucose per Kg. of body weight (Table I). A small, but statistically significant, increase in fasting venous blood sugar levels with age was observed in this sample.

Reliability of methods

The standard deviation of repeated glucose determinations on a single filtrate was ± 1.1 mg. per cent (N = 58). Comparing two filtrates, prepared from the same blood sample, the standard deviation was 2.1 mg. per cent (N = 53).

There was no systematic difference between fasting blood sugar levels determined on the same individual on different days. The standard error of estimate between measurements made on the first and second days was ± 6.5 mg. per 100 ml.[4]

In the 8 subjects (4 old and 4 middle-aged) who had duplicate glucose tolerance and glucose-insulin tolerance tests, no significant differences occurred between the results of the first and second tests, with respect to k_G or k_{GI}. The dupli-

[4] Mean values are reported with standard errors of the mean.

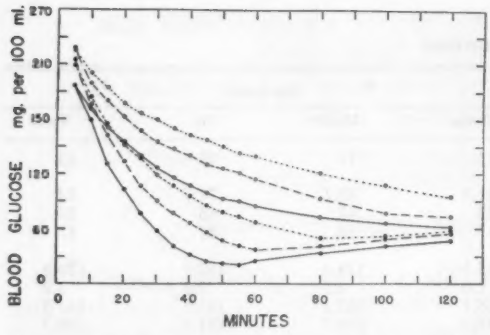

FIG. 1. LINEAR PLOTS OF AVERAGE BLOOD SUGAR VALUES (MG. PER 100 ML.) AT EACH TIME INTERVAL (MIN.) FOLLOWING THE INTRAVENOUS ADMINISTRATION OF 25 GM. GLUCOSE FOR EACH OF THREE AGE GROUPS

Glucose tolerance (GTT) open circles; glucose plus 5 units insulin per sq. M. surface area (GITT) closed circles; old subjects (65 to 87 years old) dotted lines; middle-aged subjects (42 to 58 years old) dashed lines, and young subjects (23 to 37 years old) solid lines.

cate tests were not performed in any specific order; the lapse of time between tests varied from one week to three months. In contrast to results reported by Hlad, Elrick, and Witten (32) values for k_G or k_{GI} were repeatable and characteristic for the individual.

General description of results

Average values of blood sugar concentration at each time interval, following the administration of glucose, are plotted for each of the three age groups in Figure 1. The rate of fall in blood glucose level was greater for the young than for the old subjects under both experimental conditions. When tests of significance of age differences were applied to specific time points along the glucose or glucose-insulin tolerance curves, true differences ($P < 0.001$) were found between young and old subjects at 15, 30, and 60 minutes after injection of the glucose. Differences over shorter age spans, *i.e.*, between young and middle, and middle and old subjects, were usually significant at $P < 0.01$ or $P < 0.05$.

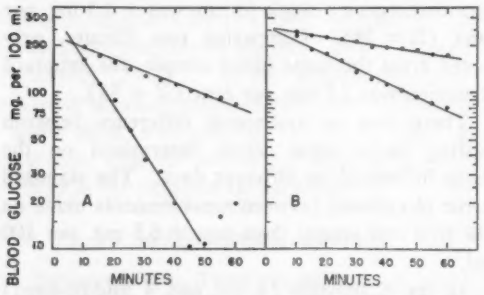

FIG. 2. ESTIMATION OF K_G AND K_{GI} IN INDIVIDUAL EXPERIMENTS

Log glucose concentration in blood (mg. per 100 ml.) plotted against time (min.). A. Young subject (26-year-old). B. Old subject (86-year-old). Upper lines (open circles) represent data from GTT; lower lines (solid circles) represent data from GITT.

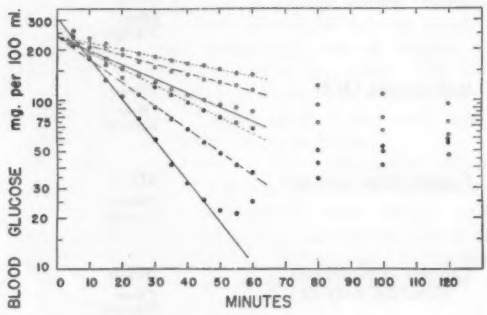

FIG. 3. AGE DIFFERENCE IN K_G AND K_{GI}

Plots of log glucose concentration in the blood (mg. per 100 ml.) against time (min.) following intravenous administration of 25 Gm. glucose. Glucose tolerance (GTT) open circles; glucose plus 5 units insulin per $M.^2$ surface area (GITT) solid circles; old subjects (65 to 87 years old) dotted lines; middle-aged subjects (42 to 58 years old) dashed lines, and young subjects (23 to 37 years old) solid lines. (Lines are fitted to points for 10 to 60 min. inclusive.)

Age differences in rate of disappearance of glucose from the blood

When the log glucose concentration was plotted against time, a linear relationship was obtained for the points between ten and 50 to 60 minutes. Sample plots for a young (26-year-old) and an old (86-year-old) subject are shown in Figure 2A and 2B. Figure 3 shows the log of the mean glucose values for the three groups of 8 subjects, plotted against time after glucose administration. Deviations from linearity are apparent in all curves beyond 50 or 60 minutes. Therefore, the expression $\log_e y = \log_e A - kt$ fails to describe the total process, but may be used to derive an index of the rate of disappearance of glucose from the

TABLE II

Blood glucose levels at specified times following intravenous administration of 25 grams glucose (G) and intravenous administration of 25 grams glucose plus 5 units per M.² of insulin (GI) in young males

Subject	Condition	Age (*yrs.*)	Wgt. (*Kg.*)	Ht. (*cm.*)	Blood sugar levels (*mg./100 ml. at specified time—min.*) 0	10	15	20	25	30	35	40	45	50	55	60	80	100	120	k (%/*min.*)
J. B.	G	32	69.1	180.3	73	152	146	126	119	105	93	80	78	77	72	67	68	65	67	1.73
	GI				81	153	123	87	58	37	19	24	22	23	28	35				8.26
F. B.	G	33	80.0	182.2	74	215	161	165	139	115	90	70	63	61	57	56	60	63	73	3.13
	GI				74	156	128	97	80	55	39	34	27	19	20	22	39	42	51	5.30
E. C.	G	36	71.4	175.3	61	190	168	154	140	129	120	112	101	96	89	85	66	55	52	1.60
	GI				65	162	97	77	54	42	14	13	6	4	7	25				9.36
L. C.	G	35	71.8	165.7	73	212	182	165	157	149	137	130	120	117	109	98	83	75	67	1.39
	GI				72	181	155	129	97	84	70	46	47	41	30	27	31	55	60	3.92
R. H.	G	32	66.0	165.7	57	205	189	171	155	135	131	116	112	112	98	95	68	51	41	1.55
	GI				67	194	142	112	83	55	40	27	13	7	2	6	27	33	40	9.30
J. J.	G	27	60.4	172.7	67	216	190	170	158	152	147	139	132	115	116	108	84	70	60	1.36
	GI				69	256	159	139	110	88	67	49	42	37	27	30	43	46	57	4.74
D. L.	G	37	71.0	176.5	60	176	166	128	107	88	68	73	71	60	68	59	56	68	59	2.81
	GI				73	142	113	82	59	38	17	17	17	23	29	29				7.80
M. N.	G	37	83.4	175.3	74	193	176	167	152	141	135	126	119	112	109	102	83	72	65	1.26
	GI				68	160	117	94	74	54	42	37	22	23	23	31	37	42	49	5.07
T. R.	G	23	57.3	171.4	65	191	169	156	135	128	119	113	102	93	85	80	58	52	49	1.70
	GI				55	153	120	94	74	52	30	25	12	12	16	18	22	28	35	6.88
B. T.	G	28	72.2	185.4	66	195	172	155	139	127	120	112	102	94	92	84	66	58	53	1.63
	GI				60	161	112	89	57	37	31	19	9	10	17	19	23	27	27	7.78
C. W.	G	25	64.8	168.9	68	202	185	186	172	161	149	137	124	120	118	110	91	73	69	1.27
	GI				73	221	162	142	105	91	76	51	48	39	35	33	50	51	59	3.85
L. Y.	G	31	85.0	184.2	73	201	185	183	176	170	165	155	147	142	139	136	123	109	97	0.78
	GI				73	175	136	93	74	55	45	37	35	29	22	21	33			4.44
Mn	G	31	71.0	175.3	67	191	171	156	141	127	116	107	99	93	90	85	73	66	62	1.68
	GI				69	179	131	103	77	58	41	32	25	22	21	25	34	41	47	6.39
SE_{Mn}	G		2.4	2.0	2.1		5.8			9.7			10.7			8.9				.19
	GI				2.0		6.1			5.8			4.3			2.3				.60

TABLE III

Blood glucose levels at specified times following intravenous administration of 25 grams glucose (G) and intravenous administration of 25 grams glucose plus 5 units per M.² of insulin (GI) in middle-aged males

Subject	Condition	Age (yrs.)	Wgt. (Kg.)	Ht. (cm.)	Blood sugar levels (mg./100 ml. at specified time—min.) 0	10	15	20	25	30	35	40	45	50	55	60	80	100	120	k (%/min.)
S. C.	G	42	65.8	165.1	78	231	209	199	184	179	164	155	144	140	131	124	103	86	73	1.21
	GI				81	216	198	171	144	126	111	96	87	77	68	65	51	51	59	2.54
C. C.	G	53	66.9	182.9	73	199	157	126	107	86	73	71	59	65	69	62	56	58	69	3.41
	GI				78	159	121	96	71	57	46	36	35	28	20	21				4.39
W. D.	G	43	61.8	186.7	92	231	215	189	170	148	149	139	130	121	115	106	73	73	76	1.49
	GI				87	213	167	136	105	93	71	63	50	46	40	37	44	50	56	3.56
J. D.	G	42	72.5	166.4	77	216	200	187	172	164	149	143	135	131	126	117	100	89	75	1.20
	GI				73	205	167	133	102	86	71	59	46	40	29	31	41	39	50	4.21
A. E.	G	58	60.9	185.4	76	224	198	185	174	167	159	158	145	138	136	128	112	96	81	1.02
	GI				85	214	192	173	146	126	107	96	81	69	58	53	39	52	45	2.91
L. G.	G	50	55.7	167.6	66	218	197	173	162	145	134	127	114	103	96	94	62	58	47	1.72
	GI				62	169	138	116	96	75	59	45	35	25	26	15	26	41	49	4.71
E. H.	G	54	55.7	170.2	70	231	226	212	208	198	187	181	170	168	157	150	129	111	86	0.87
	GI				85	199	170	128	106	98	81	71	61	46	40	40	43	51	54	3.48
D. J.	G	58	52.0	166.4	80	248	216	190	174	158	152	148	144	138	128	121	97	80	75	1.29
	GI				80	206	180	157	139	113	109	100	92	80	75	69	44	55	59	2.17
J. J.	G	44	92.7	175.3	71	193	175	167	150	139	137	128	119	115	110	106	89	80	74	1.19
	GI				86	180	142	121	91	69	74	60	48	42	32	32	59	67	72	3.49
J. R.	G	53	71.1	185.4	87	220	186	175	163	149	141	135	124	123	116	109	93	88	83	1.29
	GI					193	153	119	93	77	63	56	47	44	34	33	32	45	57	3.55
R. W.	G	43	55.1	167.6	80	249	227	214	193	188	181	166	162	152	146	138	106	87	79	1.13
	GI				70	195	147	118	77	70	55	49	37	28	25	23	39	43	49	4.67
Mn	G	49	64.6	174.4	77	223	201	183	169	156	148	141	131	127	121	114	93	78	74	1.44
	GI				79	196	162	134	107	90	77	67	56	48	40	37	42	49	55	3.61
SE_{Mn}	G		3.5	2.7	2.3		6.5			9.0			8.9			6.9			3.1	.21
	GI				2.6		8.1			7.2			6.4			5.8			2.4	.25

TABLE IV

Blood glucose levels at specified times following intravenous administration of 25 grams glucose (G) and intravenous administration of 25 grams glucose plus 5 units per M.² of insulin (GI) in aged males

Subject	Condition	Age (yrs.)	Wgt. (Kg.)	Ht. (cm.)	Blood sugar levels (mg./100 ml. at specified time—min.) 0	10	15	20	25	30	35	40	45	50	55	60	80	100	120	k (%/min.)
F. B.	G	72	68.2	143.5	80	223	195	181	162	150	144	132	127	121	116	109	94	86	77	1.36
	GI				88	193	162	143	132	120	105	99	89	77	74	68	46	51	58	2.04
T. B.	G	86	56.1	160.0	90	243	219	204	195	178	168	158	150	139	133	130	105	95	79	1.26
	GI				74	182	151	142	117	107	100	84	78	64	57	59	39	41	42	3.58
J. H.	G	73	53.5	161.3	75	211	194	180	176	171	162	155	152	148	146	158	108	108	95	0.79
	GI				65	188	150	134	120	94	83	76	65	56	51	49	39	37	42	2.74
E. K.	G	76	55.6	156.8	83	238	238	217	205	204	197	181	166	165	157	152	136	120	105	0.96
	GI				80	232	208	184	165	150	138	124	118	112	99	85	67	54	57	1.88
J. He.	G	85	70.1	162.6	95	255	223	190	184	186	181	178	174	165	155	141	142	131	114	0.91
	GI				83	202	170	153	135	121	98	86	71	69	66	60	54	63	71	2.52
W. L.	G	87	83.4	168.9	92	220	204	184	177	167	156	155	144	141	134	130	113	102	88	0.82
	GI				96	192	152	128	111	95	74	76	62	59	64	51	45	53	71	2.95
D. M.	G	84	45.1	156.2	82	278	258	236	211	206	188	182	174	163	141	141	122	100	88	1.35
	GI				73	223	193	160	142	119	108	89	75	65	54	50	36	33	39	3.06
J. M.	G	65	82.4	171.4	96	220	216	211	205	194	189	185	179	176	165	155	147	122	104	0.67
	GI				94	212	197	180	168	148	141	130	118	106	103	93	69	63	66	1.66
G. P.	G	79	60.7	171.4	86	206	194	179	176	171	166	162	158	151	149	143	127	113	101	0.67
	GI				86	186	177	154	143	131	121	109	101	94	86	80	58	49	58	1.73
D. R.	G	82	61.8	161.3	78	211	193	174	164	149	135	132	124	113	111	105	92	80	71	1.36
	GI				78	210	171	140	118	97	67	64	54	51	38	31				3.74
A. T.	G	73	77.0	167.6	101	247	232	228	209	212	198	191	192	186	176	174	149	136	123	0.68
	GI				92	231	190	168	159	135	122	113	103	94	86	81	66	66	74	2.04
L. W.	G	82	88.2	170.2	83	215	204	186	183	173	165	162	150	144	138	138	119	104	99	0.91
	GI				82	201	174	144	140	128	121	101	94	89	97	89	73	68	64	1.99
Mn	G	78	66.8	162.6	87	231	214	198	187	180	171	164	158	151	143	140	121	108	95	0.98
	GI				83	204	175	152	138	119	107	96	86	78	73	67	50	53	58	2.49
SE_{Mn}	G		3.9	2.3	2.3		6.0			6.0			5.9			5.7				.08
	GI				2.7		5.6			5.4			6.2			5.2				.02

TABLE V

Rate of disappearance of glucose from the blood with (k_{GI}) and without insulin (k_G) (k expressed as per cent per minute)

Age group	N	Venous blood: Glucose (k_G) Mn.	$\sigma_{Mn.}$	Venous blood: Glucose-insulin (k_{GI}) Mn.	$\sigma_{Mn.}$	Venous blood: Diff. (Δk) Mn.	$\sigma_{Mn.}$	Arterial blood*: N	Arterial blood*: Glucose (k_G) Mn.	$\sigma_{Mn.}$
Young	12	1.68	.19	6.39	.60	4.12	.59	13	1.94	.20
Middle	11	1.44	.21	3.61	.25	2.17	.26	13	1.61	.09
Old	12	0.98	.08	2.49	.02	1.52	.17	38	1.28	.06
Total	35	1.37	.11	4.15	.11	2.62	.09	64	1.48	.07

* Data from Smith and Shock (19) recalculated.

blood during the first 50 to 60 minutes of the experiment. Examination of Figure 3 shows that: (a) the early rate of disappearance of excess of glucose from the blood was more rapid in the young than the old, (b) the rate of disappearance was increased in all age groups by the simultaneous administration of insulin, and (c) the influence of insulin was greater in the young than the old subjects.

Computations by the method of least squares of the slope for each individual test provided data for estimates of the variability of k within each age group. Tables II, III, and IV present the values of k for both the GTT and the GITT with increasing age. Average values of k, expressed as per cent per minute, for the young and old, respectively, were as follows: k_G, 1.68 and 0.98; k_{GI}, 6.39 and 2.49, and Δ k ($k_{GI} - k_G$), 4.12 and 1.52. Values of P were less than 0.01 for these age differences. Regressions of k on age were significant at the 0.01 level for k_G and at less than 0.001 for k_{GI} and Δ k. The effect of insulin in the average adult male was to increase the rate of fall in blood glucose level from 1.37 per cent per minute to 4.15 per cent per minute.

DISCUSSION

Subject selection—Activity and diet

Since age differences in glucose tolerance are relatively small, it is necessary to give careful consideration to the selection of subjects. In order to minimize the effects of prior diet, which has been shown to have an influence on the glucose tolerance (33–36), only subjects who had been on a standard hospital diet for at least one week were tested in the present series.

Since reduced activity has been shown to reduce glucose tolerance (36, 37), only ambulatory patients were studied. The young subjects were also patients drawn from an ambulatory in-hospital population. Thus, the level of activity was probably more uniform between the different age groups than would have been the case had staff members been used for the younger age groups.

Dose of glucose and insulin

Although different amounts of glucose have been used by previous investigators, recent studies have shown that adjustment of the dose of glucose to body size is unnecessary (18, 28, 38). Consequently, a standard dose of 25 Gm. of glucose was administered at a uniform rate (18, 38) to all subjects in the present study. The glucose load varied from 0.57 Gm. per Kg. to 0.27 Gm. per Kg. in different subjects, but there were no systematic differences between age groups (Table I). The dose of insulin was set at 5 units per M.^{2}, which is approximately 0.1 unit per Kg. body weight. Experimental studies (22–24, 27, 39–41) indicate that insulin gives a maximum effect on rate response at dose levels of 0.05 unit per Kg. and up to 0.3 unit per Kg. The effect of differences in endogenous insulin production would be obliterated at this dose range of injected insulin.

Comparison of venous and arterial blood samples

Nelson's modification of the Somogyi method (30) was used for sugar determination in this study in order to minimize the effects of nonfermentable reducing substances (42, 43). The use of venous blood samples in conjunction with an

indwelling needle has the advantages of good patient acceptability and a minimum of patient trauma (44–46). Although the differences between arterial and venous blood glucose levels are small under fasting conditions (arterial bloods average 9 mg. per 100 ml. higher than venous), there is a wide range of individual variation (1 to 17 mg. per 100 ml. in normal subjects) and the difference increases markedly (average 30 to 43 mg. per 100 ml.) following the administration of glucose (43, 47). Since Blotner (37) has found that glucose tolerance determined on venous blood samples was influenced less by physical activity in both children and adults than were estimates derived from capillary blood samples, it follows that the use of venous blood might be a more adequate test of age differences in glucose tolerance.

Since previous studies of glucose tolerance from this laboratory were based on arterial blood samples (19), the data were fitted to the tolerance equation. A total of 64 subjects, divided into three age groups, were tested under conditions closely approximating the present study, except that blood samples were drawn from the femoral artery (19). Insulin was not given. Table V gives the k values based on arterial blood samples.[5] As was true for venous blood samples, there was a significant decrement in k_G with increasing age. However, the trend toward increasing fasting blood sugar levels with age, found in the present study on venous blood and in the report on capillary blood by Schneeberg and Finestone (20), was not apparent in arterial blood.

The tolerance equation

One simple expression which can serve to express the rate of disappearance of glucose from the blood, as a single number, is the equation $\log_e y = \log_e A - kt$ (48–51). If one assumes that no distinction is made between the glucose added to the blood from an external source and the glucose added by the liver or other cells of the body, the estimates of the slope of the curve must be made on the total glucose content at successive time intervals. This assumption may be applied safely only to the early parts of the curve, since it is obvious that alterations in the glucose concentration will be introduced by other processes in the body which tend to add glucose to the circulation, particularly when blood sugar levels fall to low values, as in the case when insulin is administered. Greville (50) as well as Hlad, Elrick, and Witten (32) found that subtraction of a calculated asymptotic value of blood sugar level resulted in a somewhat better fit to glucose tolerance data beyond 90 minutes. In this study where curves were limited to the first 60 minutes, the fit was very good; the introduction of an asymptote had only a small insignificant effect on goodness of fit. Furthermore, the use of an asymptote gave rise to difficulties in comparing the GTT and the GITT, since subtraction of a calculated asymptote for the glucose-insulin curves often resulted in values less than zero. Although the value of k is related to the level of the asymptote, both parameters are determined by the same set of experimental points.

In agreement with other studies (49–51), the blood glucose level at 5 minutes, following the injection, was found to be higher than predicted from the exponential curve, suggesting an interaction with the early extra-cellular mixing phase. By 10 minutes after the glucose injection, the mixing phase is indistinguishable from the body of the curve. Conard, Franckson, Bastenie, Kestens, and Kovacs (52) injected glucose and thiocyanate simultaneously and found a thiocyanate space of 14.31 L. and a glucose space of 14.01 L. In our experiments, the average glucose space was 14.1 L. or 22 per cent of the body weight. This value is only slightly lower than the glucose space (23.3 per cent of body weight) reported by Hlad, Elrick, and Witten (32) on the basis of continuous infusions of glucose. Since there are no significant changes with age in the thiocyanate space (53), it appears that the values of k are determined by the distribution of glucose in

[5] Arterial k values were based on a visual fit. A comparison between the derivations of k by least squares and graphic estimates, made from a visually fitted line using the venous data, gave mean values of 137 ($k \times 10^4$) for both methods for the GTT ($r = 0.96$). For the GITT, the mean values of k by the least squares' method was 415 as compared to 439 by the visual method ($r = 0.89$). Age did not influence the correlation between methods. Thus, a visual fit to the data yields substantially the same results as analysis by least squares. However, the latter permits a quantitative statement of the "goodness of fit" of the equation. This is not possible when the visual method above is used. Age did not influence the goodness of fit.

the intra-cellular fluids and metabolic pathways, and are influenced very little by extra-cellular mixing and not in a manner which is age biased.

Age differences

Although there is an overall reduction in the rate of removal of glucose from the blood and a reduced response to insulin with increasing age, it cannot be assumed that this reduction is necessarily associated with altered cellular metabolism. A similar overall effect could result from a reduction in the number of metabolizing units. Other studies from this laboratory have indicated a gradual loss of metabolizing tissue with increasing age (54). This conclusion is based on the observed decrease in intra-cellular water (antipyrine space minus thiocyanate space) with age. The intra-cellular space, calculated in this fashion, averages 10.5 L. in young and 8.6 L. in aged subjects; a reduction of 18 per cent. Over the same age span, k shows a reduction of 42 per cent for the glucose tolerance data and 61 per cent for the glucose-insulin tests. Although the data do not represent observations made on the same subjects, and the dimensions are incongruous, it seems difficult to account for all of the changes observed on the basis of a loss of functioning protoplasm alone.

It is conceivable that the age differences in the rate of removal of glucose from the blood might be due to differences in blood flow and delivery of glucose to the tissues. Clearly, a reduction in the total amount of blood delivered, per unit of time, would influence k if all the glucose were removed in a single passage through a vascular bed. However this is not the case. If there were a substantial reduction in blood flow to tissues in the older subject, an increase in the A-V difference should appear. Since we do not have simultaneous arterial and venous glucose levels on the same subjects, no final decision can be reached on this question, but it does not seem likely that differences in blood flow can account for the age differences observed.

It is recognized that the concentration of glucose in the blood at any given time represents an equilibrium between the rate of removal and the rate of release of glucose from the liver. The differential effect of insulin in the three age groups makes it improbable that the results obtained can be ascribed to differences in the rate of release of glucose from the liver in the old and young subjects. Recent studies indicate that the early effect of insulin action is that of increasing peripheral uptake and metabolism of glucose. The liver response is minimal and delayed (55).

Inactivation of insulin by a plasma constituent may be a factor in some phases of diabetes, particularly in regard to the mechanism of clinical insulin resistance. Welsh, Henley, Williams, and Cox (56) studied the plasma binding of insulin I^{131} in 118 subjects, 43 of which were non-diabetic ranging from 14 years to 90 years of age. Analyzing their data with reservation for the inclusion of patients with other active disease in their group of non-diabetic controls, no trend is discernible between the age of the subject and the potential insulin inactivation by plasma binding.

Although proof cannot be offered, it seems reasonable to assume that at least part of the age differences can be ascribed to alterations in the metabolic effectiveness and response of the functioning cells in the aged male.

SUMMARY

Intravenous glucose tolerance and glucose-insulin tolerance tests were performed on 35 normal male subjects under standardized conditions using venous blood samples. The subjects ranged in age from 23 to 86 years. Blood samples drawn at 5-minute intervals, between 5 and 60 minutes after administration of 25 Gm. glucose, were analyzed for glucose by the Nelson method. The rate of fall of the blood sugar level between 10 and 60 minutes was determined by fitting the experimental points to the equation $\log_e y = \log_e A - kt$. A significant decrease in k with age was observed in both the glucose and the glucose-insulin tolerance curves. The administration of insulin had a greater effect on the rate of disappearance of glucose from the blood in the young than the old subjects. It is proposed that the age difference may result from both a reduction in the amount of functioning protoplasm and an alteration in intra-cellular glucose metabolism.

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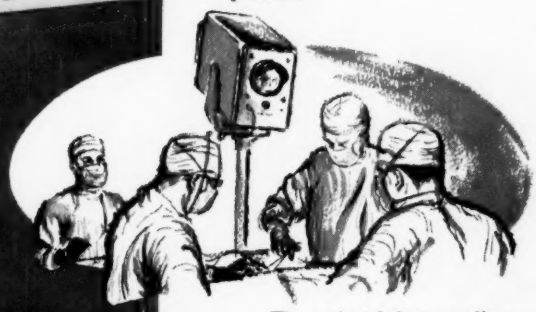

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